U.S. DEPARTMENT OF COMMERCE
National Technical Information Service

PB-267 973

A Controlled Bioassay System for Measuring Toxicity of Heavy Metals

Michigan Univ, Ann Arbor

Prepared for

Environmental Research Lab, Duluth, Minn

Apr 77

PB 267 973

EPA-600/3-77-037
April 1977

Ecological Research Series

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U.S. Environmental Protection Agency, have been grouped into nine series. These nine broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The nine series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies
6. Scientific and Technical Assessment Reports (STAR)
7. Interagency Energy-Environment Research and Development
8. "Special" Reports
9. Miscellaneous Reports

This report has been assigned to the ECOLOGICAL RESEARCH series. This series describes research on the effects of pollution on humans, plant and animal species, and materials. Problems are assessed for their long- and short-term influences. Investigations include formation, transport, and pathway studies to determine the fate of pollutants and their effects. This work provides the technical basis for setting standards to minimize undesirable changes in living organisms in the aquatic, terrestrial, and atmospheric environments.

This document is available to the public through the National Technical Information Service, Springfield, Virginia 22161.

EPA-600/3-77-037
April 1977

A CONTROLLED BIOASSAY SYSTEM FOR MEASURING TOXICITY OF HEAVY METALS

By

K. H. Nancy and H. E. Allen
Department of Environmental and Industrial Health
School of Public Health
The University of Michigan
Ann Arbor, Michigan 48109

Contract Number 14-12-591

Project Officer
Gary E. Glass
Environmental Research Laboratory-Duluth
Duluth, Minnesota 55804

ENVIRONMENTAL RESEARCH LABORATORY-DULUTH
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
DULUTH, MINNESOTA 55804

DISCLAIMER

This report has been reviewed by the Environmental Research Laboratory-Duluth, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/3-77-017	2.
3. RECIPIENT'S ACCESSION NO.	
4. TITLE AND SUBTITLE: A CONTROLLED FLOW-THROUGH SYSTEM FOR MEASURING TOXICITY OF HEAVY METALS	5. REPORT DATE: April 1977 (issuing date)
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): H. H. Nancy and H. E. Allen	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Environmental & Industrial Health, School of Public Health, The University of Michigan, Ann Arbor, Michigan 48109	10. PROGRAM ELEMENT NO. 1BA608
	11. CONTRACT/GRANT NO. Contract No. 14-12-591
12. SPONSORING AGENCY NAME AND ADDRESS: Environmental Research Laboratory-Duluth, Office of Research and Development, U.S. Environmental Protection Agency, Duluth, Minnesota 55804	13. TYPE OF REPORT AND PERIOD COVERED: Final Project Report
	14. SPONSORING AGENCY CODE: EPA/600/01

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Biological availability of metal micronutrients and metal toxicity are believed to be dependent on metal oxidation state, complexation, and solubility as well as the physicochemical characteristics of the aqueous phase. Basic design criteria for fish bioassays which are capable of elucidating the dependency of toxicity on the type and concentration of various copper species were developed utilizing equilibrium chemical concepts and appropriate analytical techniques.

In order to maintain a desired copper species in the bioassay medium, synthetic waters were used under well-defined physical and chemical conditions. These solutions were synthesized in accordance with equilibrium models, which define the distribution of various copper species as a function of the solution physical and chemical characteristics. An experimental system was developed which permitted large volumes of the bioassay waters to be maintained at the desired chemical equilibria for the duration of the experiment.

Monitoring of the bioassay system included measurements of (a) pH, (b) temperature, (c) flow, (d) specific conductance, (e) calcium, (f) total alkalinity, (g) dissolved oxygen, and (h) copper species. Novel analytical procedures were applied for the measurement and the differentiation of copper species. This included the use of anodic stripping voltammetry and potentiometric membrane electrodes.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Experimental Design Bioassay Toxicity Fishes Chemical Equilibrium Metal Complexes		06/F 06/T

18. DISTRIBUTION STATEMENT: RELEASE TO PUBLIC	19. SECURITY CLASS (This Report): UNCLASSIFIED	21. NO. OF PAGES: 18
	20. SECURITY CLASS (This page): UNCLASSIFIED	22. PRICE: PC A02 - A01

EPA Form 2220-1 (9-73)

FOREWORD

Our nation's freshwaters are vital for all animals and plants, yet our diverse uses of water---for recreation, food, energy, transportation, and industry---physically and chemically alter lakes, rivers, and streams. Such alterations threaten terrestrial organisms, as well as those living in water. The Environmental Research Laboratory in Duluth, Minnesota develops methods, conducts laboratory and field studies, and extrapolates research findings

--to determine how physical and chemical pollution affects aquatic life

--to assess the effects of ecosystems on pollutants

--to predict effects of pollutants on large lakes through use of models

--to measure bioaccumulation of pollutants in aquatic organisms that are consumed by other animals, including man

This report defines the important parameters that must be measured or controlled when heavy metals are introduced to the aquatic environment for the purpose of determining their impact on that system. The toxic response of an organism is a function of the chemical forms of metals and their availability to the organism. System parameters which regulate the responses must be studied for a complete understanding of the bioassay system.

Donald I. Mount, Ph.D.
Director
Environmental Research Laboratory
Duluth, Minnesota

ABSTRACT

Biological availability of metal micronutrients and metal toxicity are believed to be dependent on metal oxidation state, complexation, and solubility, as well as the physicochemical characteristics of the aqueous phase. Basic design criteria for fish bioassays which are capable of elucidating the dependency of toxicity on the type and concentration of various copper species were developed utilizing equilibrium chemical concepts and appropriate analytical techniques.

In order to maintain a desired copper species in the bioassay medium, synthetic waters were used under well defined physical and chemical conditions. These solutions were synthesized in accordance with equilibrium models, which define the distribution of various copper species as a function of the solution's physical and chemical characteristics. An experimental system was developed which permitted large volumes of the bioassay waters to be maintained at the desired chemical equilibria for the duration of the experiment.

Monitoring of the bioassay system included measurements of (a) pH, (b) temperature, (c) flow, (d) specific conductance, (e) calcium, (f) total alkalinity, (g) dissolved oxygen, and (h) copper species. Novel analytical procedures were applied for the measurement and the differentiation of copper species. This included the use of anodic stripping voltammetry and potentiometric membran electrodes.

The bioassay experimental system was tested by performing toxicity tests at pH 5.7, 7.0, and 8.1. Results demonstrated the feasibility of conducting bioassay experiments under conditions of chemical equilibria.

This report was submitted in fulfillment of Contract Number 14-12-591, by The University of Michigan, under the sponsorship of the Environmental Protection Agency.

CONTENTS

Foreword iii
Abstract iv
List of Figures vi
List of Tables viii
Acknowledgments ix

I Conclusions 1
II Recommendations 2
III Introduction 3
IV Aqueous Chemistry of Copper 5
V Analytical Chemistry of Aqueous Copper 32
VI Development of the Bioassay System 58
VII Design of Bioassay Experiment 69

References 88
Appendices

A Copper Equilibria Computer Program Output 96
B pH and Alkalinity Computer Program Output 103

FIGURES

No.		Page
1	Forms of Occurrence of Metal Species in Natural Waters	6
2	Activity of Cupric Ion in Equilibrium with (a) CuO(s); (b) $Cu_2(OH)_2CO_3$ at Bicarbonate Ion Concentration of 10^{-3} M; and (c) $Cu_3(OH)_2(CO_3)_2$ at Bicarbonate Ion Concentration of 10^{-3} M	25
3	Equilibrium Diagram for Copper	26
4	Stability Field Diagram for Tenorite and Malachite as a Function of pH and Total Carbonate Concentration at Various Copper Concentrations	29
5	Analytical Scheme for Anodic Stripping Voltammetric Determination of Metals in Natural Waters	44
6	Anodic Stripping Voltammetric Characterisation of Metallic Species	46
7	Cell and Electrode Arrangement for Anodic Stripping Voltammetry	48
8	The Effect of Calcium and pH on the Variation of the Peak Current During the Titration of 5 ug/ml Humic Acid with Copper	50
9	The Effect of Calcium and pH on the Variation of the Peak Current During the Titration of 5 ug/ml Humic Acid with Cadmium	51
10	Graphical Representation of Cu^{2+}-Carbonate Ion Titrations	55
11	Physicochemical and Biological Interrelationships in Bioassay Experiments	59

No.		Page
12	Partial Pressure of Carbon Dioxide and Amount of Sodium Bicarbonate (Moles/Liter) Required to Maintain Total Carbonate = 10^{-3} Molar or Total Alkalinity = 10^{-3} Equivalents/Liter	67
13	Partial Pressure of Carbon Dioxide and Amount of Sodium Bicarbonate (Moles/Liter) Required to Maintain Total Carbonate = 10^{-3} Molar or Total Alkalinity = 10^{-3} Equivalents/Liter	68
14	Change of ASV Signal for Zn^{2+}, Cu^{2+}, and Pb^{2+} with Time	71
15	Schematic Diagram of Hatchery	73
16	Schematic Diagram of Fish-Conditioning Area	74
17	Schematic Diagram of Bioassay Area	80
18	Schematic Diagram of Bioassay System	81
19	Bioassay Exposure Chamber	82

TABLES

No.		Page
1	Distribution of Soluble Copper as a Function of pH and Total Carbonate	30
2	Sensitivities of Instrumental Methods for Monitoring Metals in Water	34
3	Summary of Trace Metal-Humic Acid-Calcium Interactions as Observed During Titrations with Metal Ions	52
4	Equilibrium and Stability Constants	57
5	Random Sequence for Adding Fish to the Chambers	77
6	Random Order for Placing Fish in Bioassay Chambers	78
7	Summary of Bioassay Physicochemical Data	81
8	Summary of Fish Survival Data and Copper Measurements	84

ACKNOWLEDGMENTS

The authors wish to acknowledge the valuable contributions of the students and staff of the Environmental Chemistry Program, whose active participation was essential for the completion of this study. Special thanks are due to J. Black, D. Miniscucci, R. Roberts, T. O'Shea, D. Johnson, B. Culp, J. Twork, and R. Lasheen.

In addition, we wish to acknowledge the contributions of Dr. M. Bender, Dr. W. Matson, and Dr. P. Colby during the initial phase of this study.

The authors also wish to acknowledge the continued interest and helpful suggestions of the Project Officer, Dr. Gary E. Glass.

SECTION 1

CONCLUSIONS

It is imperative that rigorous physicochemical controls are applied in heavy metal bioassay experiments, e.g., copper toxicity to fish, in order to elucidate the dependency of toxicity on the type and concentration of given metal species. Equilibrium chemical concepts can be used to prescribe appropriate controls which must be verified by appropriate analytical techniques.

Novel analytical procedures for the differentiation of copper species were applied utilising anodic stripping voltammetry and potentiometric membrane electrodes. These procedures are also applicable for trace metal characterisation in natural and waste waters.

Anodic stripping titration techniques were used to assess the competitive interaction between trace metals (e.g., Cu, Pb, and Cd) and alkaline earth metals (e.g., Ca and Mg) for organic complex formation. Our findings showed that copper exhibits higher tendency for organic complex formation in comparison to other trace metals. Copper affinity for organic matter is not affected by high levels of calcium in contrast to other trace metals, e.g., Cd. Understanding this type of metal interaction provides an insight into metal transformations in the aquatic environment and their toxicity effects.

SECTION II

RECOMMENDATIONS

Although water quality standards give tolerance limits in terms of total metal concentrations, the toxicity of different metal species should be ascertained. This information is essential for the development of toxicity models including physical, chemical, and biological interactions. These toxicity models may be able to predict the toxic effect of certain metal species on a given aquatic organism under specified environmental conditions.

Generally, current metal bioassay practices do not take into account metal oxidation states, complexation, and solubility. It is therefore recommended that metal bioassay experiments be conducted under well-defined physicochemical characteristics of the bioassay medium.

It is recommended that adequate analytical measurements be made so that meaningful bioassay experiments can be conducted. Such measurements are required to insure that the added toxicant is present in the bioassay medium at the specified level, and to define the physical and chemical characteristics of the medium.

In view of the increasing awareness of the complexity of chemical transformations in the aquatic environment, it is recommended that efforts be made to develop reliable analytical systems capable of metal species characterization. Such analytical tools are essential for the understanding and prediction of toxicity to aquatic biota.

SECTION III

INTRODUCTION

Fish bioassays are perhaps the oldest known procedures for determining water "safety" and for detecting harmful toxicants. The use of fish to indicate the presence of polluting materials is similar to the 19th century practice of using canaries to monitor the presence of toxic gases in coal mines. In Europe the toxicity of waste effluents is commonly monitored by placing a known number of fish in a net or screen box in the effluent stream or the receiving water and observing their survival at daily intervals. Consequently, fish bioassay tests can be considered as nonspecific analytical procedures that use fish as in situ biological sensors.

At the present time water quality criteria and standards are based largely on data from bioassay and toxicity tests with fish and other organisms. In fact, acute and chronic toxicity experiments are being increasingly used to establish guidelines for "safe" levels of toxicants and other physical and chemical characteristics of the aquatic environment. Because of the complexity of environmental factors of most natural aquatic systems, laboratory bioassay experiments, which permit the maintenance of controlled conditions, are frequently preferred over field experiments to study the effect of a given toxicant. Nevertheless, the ultimate proof of results of laboratory bioassay experiments must come from studies on natural aquatic communities.

In this report we describe procedures for conducting controlled laboratory bioassays. The main thrust of these studies has been the provi-

sion of rigorous physical and chemical controls of the bioassay experiment. Only under these conditions can the biological response be quantified and understood. The unique aspect of the work was the attempt to determine the toxicity of different forms of the toxic element, rather than the effect of total concentration per se. Thus, much effort was devoted to understanding the aqueous chemistry of copper and the development of analytical procedures for characterizing the various metal species. We hope that the reported studies will offer a fresh outlook on the toxicity of heavy metals to fishes and will aid in improving the design of bioassay studies.

The role of heavy metals in water quality and their pollutional effects on lakes, estuaries, and marine environments have received national attention. Metals such as copper, cadmium, zinc, and cobalt, frequently found in natural waters in the parts-per-billion range, may serve as essential micronutrients for enzymatic transformations in trace quantities and may exert toxic effects on the aquatic biota at higher concentrations. Lead and mercury, in particular, are toxic at certain concentrations and are considered to be direct signs of man-made pollution.

The effect of trace metals on the quality of natural waters is dependent on the distribution dynamics of these metals in the aquatic environment and the types of interactions with the aquatic habitat. Meaningful investigations should be based on the characterization of the types and transport dynamics of metals in solution, whether they are adsorbed on suspended particulate matter, bound by or released from bottom sediments. The physicochemical characteristics of the aqueous phase and the availability of organic ligands of natural or pollutional origin greatly influence the distribution and effect of trace metals on aquatic biota.

The establishment of tolerance limits and water quality standards has been based on the assumption that the availability of metal micronutrients and the toxicity of metals to biological systems are solely depen-

dent on metal concentration. This assumption is not valid, since the availability of metal micronutrients to biological systems and metal toxicity effects are highly dependent on the degree and type of metal complex formation. The biological response to trace metals in aquatic environments is greatly influenced by the presence of metal-binding ligands of natural or pollutional origin and the form of the metal species, that is, whether they are in solution or in the form of colloidal particulates.

Metals in aqueous environments are conceived to exist in a variety of forms. To differentiate simply between soluble and insoluble metals is not easy. Workers in the field frequently adopt an operational definition of "soluble" and "insoluble" metals by using 0.45u or 0.1u membrane filters.

Metal species in aqueous systems can be categorised as follows:

I. Soluble species
   1 - Free ions
   2 - Metal complexes with organic or inorganic ligands

II. Insoluble particulates
   1 - Colloidal particulates of metal complexes or aggregates of hydrous metal oxides
   2 - Metal complexes adsorbed on suspended particulates

An illustration of size distribution and the variety of species in the soluble, colloidal, and particulate range is shown in Figure 1, reported by Stumm and Bilinski [1]. For example, soluble metals can exist as (a) inorganic complexes and ion pairs, with the ligands such as H_2O, OH^-, O^{2-}, HCO_3^-, CO_3^{2-}, Cl^-, HPO_4^{2-}, PO_4^{3-}, SO_4^{2-}, HS^-, S^{2-}, etc.; (b) organic complexes with amino acids, polysaccharides, amino sugars, fatty acids, organophosphorus compounds, porphyrin, etc., and (c) polynuclear complexes.

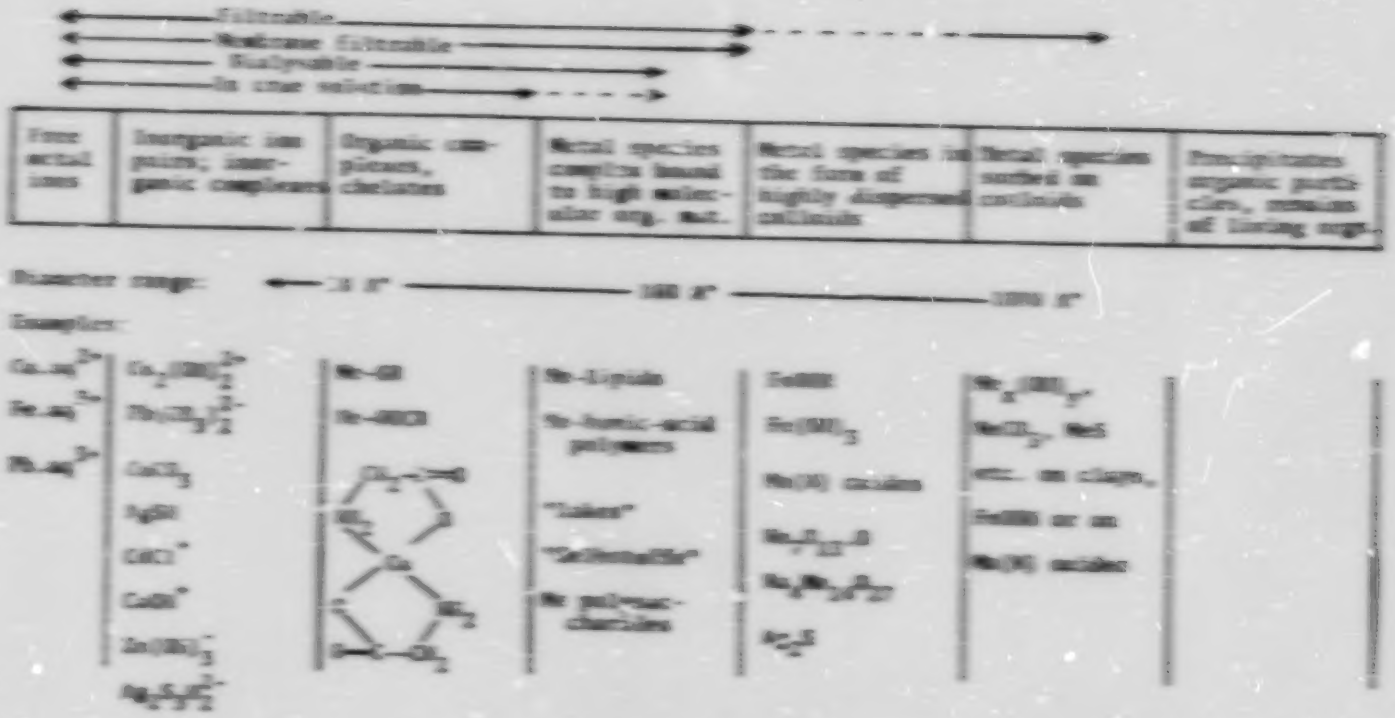


Figure 1. Forms of occurrence of metal species in natural waters[1].

Metal organic interactions have been mentioned repeatedly in recent literature to explain anomalous metal distribution that cannot be accounted for by known thermodynamic data [2,3,4,5,6,7,8]. Certain organic constituents have been postulated to complex with certain metals, and as a result the availability or toxicity of certain metals to biological systems is altered. Needless to say, some of these deductions have been substantiated by the fact that a great many metal species in natural waters are nonextractable with suitable reagents, nonseparable by ion exchangers, and nondialysable.

The literature presents diverse opinions on the subject of aquatic metal distribution and organic-metal interactions. Corcoran and Alexander[9] provided indirect evidence for the existence of trace metal organic complexes in sea water by employing perchloric acid digestion. More direct evidence was provided by dialysis and neutron activation analysis experiments performed by Slowey and Hood[10] and Slowey and Jeffrey[11]. More recently, Williams[12] has demonstrated the existence of organic copper complexes in sea water, and evidence for the existence of organic complexes with other trace metals, particularly iron, has been reported by Koshy and Ganguly[13] and Koshy, Desai, and Ganguly[14]. Duursma and Sevenhuysen[2], on the other hand, found that trace metal-organic interactions are rather insignificant. Recent radio tracer studies indicate that certain organic compounds (leucine) exhibit no chelation with trace metals at the levels normally present in the sea[15]. The existence of trace metal-organic complexes in fresh water has been suggested by the anodic stripping studies of Barsdate and Matson[16], Matson and Roe[17], and Allen, Matson, and Nancy[18]. Barsdate[19] provided further evidence through dialysis studies.

The biological importance of trace metals chelated by organic substances has also received attention. DeKock[20] showed that iron became readily available to plant cells and stimulated growth in the presence of humic

acids. This evidence of the stimulatory effect of chelated iron-humic acid substances was later used by Prakash and Rashid[21] to partially explain the increase in growth of marine dinoflagellates in the presence of humic acids. Johnston[22] found that the addition of a mixture of iron, manganese, zinc, copper, and cobalt chelated with EDTA at pH 7-8 induced abundant growth of unialgal Skeletonema costatum in most sea waters enriched with phosphate, nitrate, and silicate. Jones[23] found that Escherichia coli does not grow in sea water enriched with glucose, ammonium, and phosphate ions unless the water is treated with chelating agents. The inhibitory effect was attributed to the toxicity of heavy metal ions present in the water. Enhancement of biological growth in the presence of chelating agents, such as EDTA, has been further substantiated by Barber and Ryther[24], who found that the presence of chelators increased phytoplankton growth in the Cromwell Current. Recently, the effect of nitrilotriacetic acid, a strong chelator, on the growth of estuarine phytoplankton has been reported by Erickson, Maloney, and Gentile[25].

Although the literature suggests that some aquatic organisms can selectively concentrate certain metal ions by secreting certain chelating agents, there is no direct evidence in support of the predominance of such trace organometallics in the aquatic environment. It is not quite understood how organic matter in trace quantities can selectively interact with individual heavy metals, e.g. Cu and Zn, in the presence of a large excess of alkaline earth metals, e.g. Ca and Mg[26]. The question takes on added significance in view of the toxicity studies of Cairns and Scheier[27], Wurts[28], Pickering and Henderson[29], and Mount[30]. These workers found that the toxicity of zinc and copper to bluegills, freshwater molluska, and minnows was less in hard water than in soft water. Although the mechanism of this inhibition is not known, chemical interactions of the alkaline earths and trace organics cannot be overlooked.

In summary, considerable evidence indicates that trace metals in natural waters are, to a certain extent, present as complex species associated with trace organic materials. Further evidence is available to relate these complex organometallics to biological activity and the toxic effects of trace metals in natural media. However, little information is available on the dynamics of trace metals in natural aquatic media and on the distribution and characterization of the organometallics associated with these trace metals. Furthermore, the role of alkaline earth ions in the complexation of these trace metals is presently only a matter of speculation.

TOXICITY OF METALS TO FISH

Chen[31] discussed in his review how metals act in several ways as poisons to fish. The metals enter the organism in either of two ways -- through the gills while respiring or through the mouth with swallowing. The entrance of ionic poisons into the gills may be aided by the excretion of external enzymes to facilitate the active transport of ions against a concentration gradient. Then, the gill epithelium is destroyed by the action of certain heavy metals. Therefore, the fish dies of asphyxiation.

Toxic metals may travel to different parts of the body via the bloodstream. The poisons may react with cell protoplasm of various tissues or organs. Insofar as copper toxicity is concerned, a lack of agreement exists concerning the actual "safe level" concentration in water [32]. Copper concentrations varying from 0.1 to 1.0 mg/l have been found by various investigators to be toxic. On the other hand, concentrations of 0.015 to 3.0 mg/l were reported as toxic, particularly in soft water, to many kinds of fish.

EFFECTS OF CHELATORS ON TOXICITY

Various investigators seem to disagree on the influence of chelation on metal toxicity to fish. For example, a study of zinc toxicity to

fish by Lloyd[33] indicated that suspended zinc sulfate complexes decreased the survival time of rainbow trout in concentrated solutions. Fitzgerald[34] showed that citric acid chelation reduced toxicity of copper to minnows 500-fold. Grande[35] in his investigations with copper in several Norwegian lakes showed that one lake heavily colored with humic colloids was less toxic to salmonid fishes than the non-colored lakes, even though both lakes contained the same copper concentrations. This phenomenon was described as a masking effect by the organics on the metal. Masking effect refers to the organics' ability to reduce or "hide" the toxicity of copper to fish. This apparently depends upon the type of metal complex present in the water system which in turn determines the metal's toxicity to organisms.

Nielsen and Wium-Andersen[36] in their investigations of copper toxicity to plankton found that various organic substances also mask the effects of metals on algae. The organics appear to be polypeptides and other similar substances. Duursma and Sevenhuysen[5] determined that other organics (phenols, petroleum, and carbon disulfide) present in the natural system reduced the amount of free copper in sea water.

It is apparent from these investigations that organic chelators definitely affect free copper concentrations in the aqueous system. Whether the chelators enhance or detract from metal toxicity is still uncertain.

SOURCES OF CHELATING AND SEQUESTERING AGENTS

The major chelating and sequestering agents found in the aquatic environment include plant products, microbial metabolites, humic compounds, lipid soluble fractions, detergents, and sewage effluent. These sources of complexing agents are discussed below.

In a review by Pauli[37] concern was given to the exogenic cycle of matter and the manner of occurrence and distribution of minerals in the outer surface of the earth. The most effective method of transporting

elements from the lithosphere to the biosphere, including the fresh water cycle, was reported to involve the biosynthesis of chelating and sequestering agents. These agents were classified as plant products, microbial metabolites, or humic compounds. Chelating agents produced by organisms include lichen acids such as lakmus and orcein, which are plant-derived polyphenols with two or more aromatic rings.

Humic compounds are derived from decomposition of organic residues through biological activity and heterogeneous catalysis. The humic compounds are collectors of copper, zinc, nickel, cadmium, lead, manganese, and cobalt. These chelates formed from humic substances and trapped metals become available for use by various organisms in the terrestrial and fresh water biocycle.

Slowey and Jeffrey[11] experimented primarily with copper complexes found in sea water. The largest amounts of extractable copper were found at depths where sinking, decaying organisms were most likely to release organic compounds into sea water. Rupture of the phytoplankton and zooplankton releases some of the organic complexing compounds to the water. Although the nature of these complexes is not resolved, some evidence indicates that the copper complex is associated with the phospholipid, amino lipid, or porphyrin fraction of these lipids. Although these studies concern sea water, fresh waters probably contain similar complexing organics.

Williams[12] demonstrated that organically associated copper may be complexed with phospholipids, carotenoids, or other lipid-soluble fractions. However, it is not clear whether the copper is actually a molecular complex with an organic ligand or whether it is associated with organic colloids by bonding within an organic matrix.

Detergents are obviously a significant component of domestic sewage. According to Hynes[38], raw sewage contains about 12 ppm of active detergent. A significant proportion of detergents remains undegraded and eventually gets into water bodies. Detergent builders are strong complexing agents which must be considered in studies of ligands in natural waters.

EFFECT OF ALKALINE EARTH METALS ON METAL TOXICITY

A first observation of the metallic content of natural water reveals a predominance of alkalies and alkaline earth metals. Stumm and Morgan [35] were therefore prompted to pose the following questions: (1) How can trace concentrations of organic matter specifically interact with individual metals? and (2) Does not the presence of Ca^{2+} and Mg^{2+} at concentrations many orders of magnitude larger than potential organic complex-forming species blur any complex-forming tendency of the organic functional groups? These pertinent questions cannot be answered from present available information on trace metals in the aquatic environment.

The effect of high concentrations of alkaline earth metals on transition or heavy metal-organic complex formation can be illustrated by considering the effect of Ca^{2+} and Mg^{2+} on EDTA complex formation with Fe(III). The formation of Fe(III)-EDTA complexes is given as

$$Fe^{3+} + Y^{4-} = FeY^{-}; \quad \log K_{FeY} = 25.1 \tag{1}$$

The effect of Ca^{2+} can be expressed as

$$CaY^{2-} + Fe^{3+} = FeY^{-} + Ca^{2+}; \quad \log \frac{K_{FeY}}{K_{CaY}} = 14.4 \tag{2}$$

where $\log K_{CaY} = 10.7$.

Based on the above relationships, FeY^{-} cannot be formed to any significant extent if the ratio of $[Ca^{2+}]$ to $[Fe^{3+}]$ is sufficiently high. This effect is of course dependent on the relative stability of the formed complexes. For example, in the case of L = citrate^{4-} the equilibrium

$$CaL^{-} + Fe^{3+} = FeL + Ca^{2+} \tag{3}$$

is characterized by an equilibrium constant of $\log (K_{FeL}/K_{CaL}) = 7.4$. This indicates that the competitive effect of Ca^{2+} upon Fe^{3+} complexing by citrate is much smaller than that encountered with EDTA.

Numerous investigators have studied the effect of hardness by measuring the toxicity of metal solutions with increasing hardness, and several theories have been advanced to account for their results. Early workers attributed the decreased toxicity of solutions at high hardness to complexation and precipitation of the metal ions from the solution. However, with the advent of continuous-flow bioassay techniques, it has been found that some complexation may actually increase the toxicity of metal solutions[30]. Mount's study showed that at any given hardness level, as pH increased, the solutions became more toxic. He attributed this effect to the zinc complexes formed at higher pH levels.

One of the major difficulties in interpreting the effect of hardness on heavy metal toxicity stems from the inability to determine whether the observed effects are a result of calcium-heavy metals competitive interactions or are due to physiological effects that resulted from the presence of Ca^{2+} or Mg^{2+}, or both. For example, the effect of hardness could be explained in terms of purely aqueous chemical interactions. Under a given set of conditions, certain heavy metals can exist as soluble organic complex species. At relatively high Ca^{2+} concentrations, as a result of metal-ligand exchange reactions, these heavy metals may be transformed into inorganic metal complexes that may precipitate as hydroxy compounds at high pH. This effect will be more pronounced at higher levels of "hardness" and of course is largely dependent on the pH. Consequently, if the toxicity effects were primarily due to the organic-metal complex, the presence of high levels of calcium will result in a decrease of its toxicity.

On the other hand, the presence of high levels of Ca^{2+} and Mg^{2+} has been thought to cause certain physiological changes in fishes that may result in a decrease in the toxic effects of heavy metals. This was shown by Lloyd[33], who found that preconditioning of fish in hard water will significantly decrease the animal's susceptibility to toxic metals.

SECTION IV

AQUEOUS CHEMISTRY OF COPPER

The presence of copper in water is ecologically significant because of its extreme toxicity to fish and other aquatic organisms. Workers have studied the toxicity of copper, but none have investigated the relationship between the speciation of copper and its toxicity. A comprehensive study of the toxicity of copper is required to provide the information necessary for evaluating its environmental impact.

SOURCES

Most of the copper entering the aqueous environment is due to the activities of man. Natural weathering of minerals and the resulting runoff, which contains copper, contribute an almost insignificant fraction of the total copper input. The copper washed out of the atmosphere by precipitation is significant. Lazrus, Lorange, and Lodge[39] found an average of 21 µg/l Cu in precipitation in the United States. Kramer[40] found an average of 32 µg/l total copper and 22 µg/l soluble copper in precipitation collected at 16 stations in northern Ontario. Although the copper reaching bodies of water by precipitation is a natural occurrence, a large fraction of the copper in the atmosphere is not of natural origin.

Large amounts of copper enter the natural water system from mining operations and metallurgic processes. The waste water from copper-bearing acid mine drainage is usually stored in tailings ponds after neutralising the acidity. However, the treated water in the tailings pond may contain concentrations of copper as high as 30 µg/l[41]. The

drainage from mine mines may have pH values as low as 2.5. The acidity of the drainage water has been attributed to the metabolic activity of certain bacteria, which enhance the oxidation rate of sulfide particles by a factor of about 2,000 at the optimum temperature[42]. Prokovich[43] found concentrations of copper in water near old mines to be as high as 1,000 mg/l.

Treatment of water with copper sulfate for control of algal growth has been practiced since about 1900[44,45,46]. In some cases lakes have been treated with as much as 1.5 million pounds of copper sulfate over a period of several decades[47].

FORMATION OF COMPLEXES

The results from many studies indicate that copper in both fresh water and sea water is held in solution by complexation with naturally occurring organic ligands. Allen et al.[18] added 13 µg/l of copper to water from the Rouge River. Results of analysis using anodic stripping voltammetry indicated a logarithmic decrease in peak current with time. The results were interpreted as a first-order complexation reaction of copper and excess organic ligands in the river water.

Stability constants for copper hydroxide, oxide (tenorite), and basic carbonates (azurite, $Cu_3(OH)_2(CO_3)_2$; malachite, $Cu_2(OH)_2CO_3$) indicate that the equilibrium concentration of copper in water with pH greater than 7 would be very low. In some waters, however, the amount of copper apparently in solution exceeds theoretical limits by several orders of magnitude. This anomaly has also been explained as a result of complexation of copper with organic substances called yellow acids, humic acids, and fulvic acids. Up to 50% of the copper in sea water can be extracted with chloroform[11] indicating that much of the copper in sea water is in some organic complex or sol. Williams[12] found that 5 to 20% of the copper in sea water was present as complexes with stability constants greater than 10^{18}.

Studies concerned with the chemistry of copper in soil have also indicated that copper forms complexes with naturally occurring ligands. If solutions of copper are stirred with ion exchange resins in both the presence and absence of humic and fulvic acid the resin will take up less copper when humic or fulvic acid is present[46].

PRECIPITATION

In the absence of organic ligands, copper is very insoluble at the pH values found in most natural bodies of water (pH > 7). When soluble salts of copper ($Cu(NO_3)_2$, $CuCl_2$, $CuSO_4$) are mixed with natural water the cupric ion precipitates as copper hydroxide ($Cu(OH)_2$) and will evolve to tenorite (CuO) or to a basic carbonate, either malachite ($Cu_2(OH)_2CO_3$) or azurite ($Cu_3(OH_2)(CO_3)_2$), depending on the carbonate and copper concentration.

COPPER IN FRESH WATER AND SEA WATER

Allen et al.[18], using anodic stripping voltammetry, found copper concentrations of 19 to 108 ug/l in Rouge River water, 1.8 to 6.8 ug/l in Lake Erie, and 0.4 to 19 ug/l in Lake Michigan. Samples were acidified before analysis to release copper from organic and inorganic complexes. Analyses without prior acidification gave much lower results indicating that most of the copper present was either in complexed or particulate forms.

The copper cycle in three Connecticut lakes was described by Riley[49]. In all of the lakes studied both total and particulate (> 0.6 μ) copper concentrations reached a sharp maximum by November. The minimum concentrations in all defined forms of copper occurred from February to September.

Copper in sea water exists primarily in nonionic forms, and its vertical distribution is sharply defined[50]. A peak concentration of over 20 ug/l occurs near the base of the euphotic zone. The concentration decreases with depth into the aphotic zone to about 10 ug/l, after which it stays nearly constant with increasing depth.

SEDIMENTS

In many lakes the concentration of copper in sediments is quite high. The sediments have served as a sink for the copper that has been added to lakes for algal control. Detailed profiles of copper in the sediment of several lakes are reported by Sanchez-Vazques[47]. In all cases the copper concentration was at a maximum in the top layers of sediment, indicating that most of the copper is from the activities of man.

WATER QUALITY CRITERIA

The limit set for copper according to U.S. Public Health Service has been changed twice. The original standard, set in 1925, was 0.2 mg/l. In 1942 the limit was raised to 1 mg/l, and in 1962 it was lowered to the present standard of 1 mg/l. The toxicity of copper to human beings is low, but the present standard was set because of the poor taste that copper imparts to water. However, because copper is highly toxic to many organisms the threshold concentration of copper is reported by McKee and Wolf[32] to be 0.1 mg/l in water used for irrigation. For fish and other aquatic life the desirable concentration limit is 0.02 mg/l in soft water and 0.05 mg/l in sea water.

EQUILIBRIUM MODEL OF Cu^{2+}-H_2O-CO_2 SYSTEMS

Of particular interest to the study of copper toxicity is the development of an equilibrium model describing the homogeneous as well as the heterogeneous equilibrium distribution of copper between the insoluble and soluble hydroxy and carbonate complexes. The equilibrium relationships for prediction of the composition of an aquatic system are most easily solved by numerical digital computer techniques.

A Cu^{2+}-H_2O-CO_2 equilibrium system has been studied in our laboratory in an attempt to illustrate the chemical relationships that must be considered in the interpretation of copper toxicity data. However, great care should be exercised in extending this equilibrium approach

to the prediction of metallic species present in natural waters. Although it is possible to measure the concentrations of inorganic complexing agents such as hydroxide, carbonate, bicarbonate, sulfate, chloride, and ammonia and to incorporate these into equilibrium calculations, prediction of the effect of the organic constituents is not possible at the present time.

In the ternary system Cu^{2+}-H_2O-CO_2 we are concerned with

(a) the solid phase

$CuO_{(s)}$ (tenorite)

$CuCO_3 \cdot Cu(OH)_{2(s)}$ (malachite)

$(CuCO_3)_2 \cdot Cu(OH)_{2(s)}$ (azurite)

and with (b) the aqueous phase

H^+, OH^-, $CO_2(H_2CO_3^*)$, HCO_3^-, CO_3^{2-}, Cu^{2+}, $CuOH^+$, $Cu_2(OH)_2^{2+}$, $Cu(OH)_3^-$, $Cu(OH)_4^{2-}$, $CuCO_3$, and $Cu(CO_3)_2^{2-}$

The following equilibria relationships occur in the copper-carbonate system[26]:

	Log K	
$CuO_{(s)} + 2\ H^+ = Cu^{2+} + H_2O$ (tenorite)	7.63	(4)
$Cu_2(OH)_2CO_{3(s)} + 4\ H^+ = 2\ Cu^{2+} + 3\ H_2O + CO_{2(g)}$ (malachite)	14.16	
or		
$Cu_2(OH)_2CO_{3(s)} + 3\ H^+ = 2\ Cu^{2+} + 2\ H_2O + HCO_3^-$	6.34	(5)
$Cu_3(OH)_2(CO_3)_{2(s)} + 6\ H^+ = 3\ Cu^{2+} + 4\ H_2O + 2\ CO_{2(g)}$ (azurite)	21.24	
or		
$Cu_3(OH)_2(CO_3)_{2(s)} + 4\ H^+ = 3\ Cu^{2+} + 2\ H_2O + 2\ HCO_3^-$	5.60	(6)
$Cu^{2+} + H_2O = CuOH^+ + H^+$	- 8	(7)
$2\ Cu^{2+} + 2\ H_2O = Cu_2(OH)_2^{2+} + 2\ H^+$	-10.95	(8)
$Cu^{2+} + 2\ CO_3^{2-} = Cu(CO_3)_2^{2-} + 2\ H^+$	10.01	(9)
$Cu^{2+} + CO_3^{2-} = CuCO_{3(aq)}$	6.77	(10)
$Cu^{2+} + 3\ H_2O = Cu(OH)_3^- + 3\ H^+$	-26.3	(11)
$Cu^{2+} + 4\ H_2O = Cu(OH)_4^{2-} + 4\ H^+$	-39.4	(12)
$H_2O + CO_2 = H_2CO_3$	- 1.5	(13)
$H_2CO_3 = H^+ + HCO_3^-$	- 6.37	(14)

$$HCO_3^- = H^+ + CO_3^{2-} \qquad -10.25 \qquad (15)$$

$$CO_2 + H_2O = HCO_3^- + H^+ \qquad -7.82 \qquad (16)$$

From relationships 13, 14, and 15 it is possible to solve for $[HCO_3^-]$ and $[CO_3^{2-}]$ in terms of $[H^+]$ and $[H_2CO_3]$:

$$[HCO_3^-] = \frac{[H_2CO_3]k_1}{[H^+]} \qquad (17)$$

$$[CO_3^{2-}] = \frac{[H_2CO_3]\ k_1k_2}{[H^+]^2} \qquad (18)$$

The expression for total carbonate is:

$$[\text{Total Carbonate}] = C_T = [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}]$$

or

$$C_T = [H_2CO_3] + \frac{[H_2CO_3]\ k_1}{[H^+]} + \frac{[H_2CO_3]\ k_1k_2}{[H^+]^2} \qquad (19)$$

This allows us to define the α values as:

$$\alpha_0 = \frac{[H_2CO_3]}{C_T} = \frac{[H^+]^2}{[H^+]^2 + k_1[H^+] + k_1k_2} \qquad (20)$$

$$\alpha_1 = \frac{[HCO_3^-]}{C_T} = \frac{k_1[H^+]}{[H^+]^2 + k_1[H^+] + k_1k_2} \qquad (21)$$

$$\alpha_2 = \frac{[CO_3^{2-}]}{C_T} = \frac{k_1 k_2}{[H^+]^2 + k_1[H^+] + k_1 k_2} \qquad (22)$$

This permits the calculation of $[H_2CO_3]$, $[HCO_3^-]$, and $[CO_3^{2-}]$ if we know the pH and the total carbonate concentration.

In the $Cu-H_2O-CO_2$ system there are two distinct equilibrium situations at any particular pH and carbonate concentration. The first occurs when the $[Cu^{2+}]$ is not large enough to cause precipitation, and the second when the $[Cu^{2+}]$ is large enough to cause the precipitation of tenorite, malachite, or azurite. These two situations will be discussed separately.

Case I: No Solid Species Formed

Expressions for each of the soluble species can be obtained be rearranging equilibria relationships 7, 8, 9, 10, 11, and 12:

$$[CuOH^+] = \frac{[Cu^{2+}](10^{-8})}{[H^+]}, \qquad [Cu_2(OH)_2^{2+}] = \frac{(10^{-10.95})[Cu^{2+}]^2}{[H^+]^2}$$

$$[Cu(CO_3)_2^{2-}] = (10^{10.01})[CO_3^{2-}]^2[Cu^{2+}], \quad [CuCO_3] = (10^{6.77})[CO_3^{2-}][Cu^{2+}]$$

$$[Cu(OH)_3^-] = \frac{(10^{-26.3})[Cu^{2+}]}{[H^+]^3}, \qquad [Cu(OH)_4^{2-}] = \frac{(10^{-39.4})[Cu^{2+}]}{[H^+]^4}$$

An expression for total copper concentration is:

$$[\text{Total Copper}] = [CuOH^+]+[Cu(CO_3)_2^{2-}]+[CuCO_3]+[Cu(OH)_3^-]+[Cu(OH)_4^{2-}]+[Cu^{2+}]$$

This can also be written as:

$$[\text{Total Copper}] = \frac{[Cu^{2+}](10^{-8})}{[H^+]} + (10^{10.01})[Cu(CO_3)_2^{2-}][Cu^{2+}]$$

$$+(10^{8.77})[CO_3^{2-}][Cu^{2+}] + \frac{(10^{-26.3})[Cu^{2+}]}{[H^+]^3} + \frac{(10^{-19.4})[Cu^{2+}]}{[H^+]^4} + [Cu^{2+}] \quad (23)$$

These expressions neglect the dimeric $Cu_2(OH)_2^{2+}$, which would be significant only at much higher concentrations than those present in natural waters.

We can now define β_0 as $\beta_0 = [Cu^{2+}]/[\text{Total Copper}]$, which is equal to:

$$\beta_0 = \frac{1}{\frac{(10^{-8})}{[H^+]} + (10^{10.01})[CO_3^{2-}]^2 + (10^{8.77})[CO_3^{2-}] + \frac{(10^{-26.3})}{[H^+]^3} + \frac{(10^{-19.4})}{[H^+]^4} + 1} \quad (24)$$

This is a significant equation because it means that the cupric ion concentration, Cu^{2+}, can be calculated by knowing the pH, carbonate concentration, and the total copper concentration as follows:

$$[Cu^{2+}] = \beta_0[\text{Total Copper}]$$

where $\beta_0 = [Cu^{2+}]/[\text{Total Copper}]$ as defined above.

If we define Denom as,

$$\text{Denom} = (10^{-8})/[H^+] + (10^{10.01})[CO_3^{2-}]^2 + (10^{8.77})[CO_3^{2-}]$$

$$+ (10^{-26.3})/[H^+]^3 + (10^{-19.4})/[H^+]^4 + 1 \quad (25)$$

then similar equations can be constructed that can be solved for the concentration of each of the copper species.

The resulting δ values are:

$$\delta_0 = [Cu^{2+}]/[\text{Total Copper}] = 1/(\text{Denom}) \quad (26)$$

$$\delta_1 = [CuOH^+]/[\text{Total Copper}] = (10^{-8})/[H^+](\text{Denom}) \quad (27)$$

$$\delta_2 = [Cu(OH)_2^-]/[\text{Total Copper}] = (10^{-26.3})/[H^+]^2(\text{Denom}) \quad (28)$$

$$\delta_3 = [Cu(OH)_3^{2-}]/[\text{Total Copper}] = (10^{-39.5})/[H^+]^3(\text{Denom}) \quad (29)$$

$$\delta_4 = [CuCO_3]/[\text{Total Copper}] = (10^{6.77})[CO_3^{2-}]/(\text{Denom}) \quad (30)$$

$$\delta_5 = [Cu(CO_3)_2]/[\text{Total Copper}] = (10^{10.01})[CO_3^{2-}]^2/(\text{Denom}) \quad (31)$$

From these equations:

$$[Cu^{2+}] = \delta_0[\text{Total Copper}] \quad (32)$$

$$[CuOH^+] = \delta_1[\text{Total Copper}] \quad (33)$$

$$[Cu(OH)_2^-] = \delta_2[\text{Total Copper}] \quad (34)$$

$$[Cu(OH)_3^{2-}] = \delta_3[\text{Total Copper}] \quad (35)$$

$$[CuCO_3] = \delta_4[\text{Total Copper}] \quad (36)$$

$$[Cu(CO_3)_2^{2-}] = \delta_5[\text{Total Copper}] \quad (37)$$

Case II: Solid Species Present

In the presence of a solid copper species, whether tenorite, malachite, or azurite, the cupric ion concentration at equilibrium will be dependent on the pH, total carbonate, and solid species present.

Figure 2 shows the concentration of cupric ion in equilibrium with tenorite, malachite, and azurite in a bicarbonate concentration of 10^{-3} M. At any particular pH and bicarbonate level, the precipitate that dictates the lowest cupric ion concentration will be the predominant solid species. Tenorite will be the predominant form at pH values greater than 6 and a bicarbonate concentration of 10^{-3} M (Figure 3).

Once the solid species has been designated, the cupric ion concentration will be dictated by that particular solid species:

Tenorite: $$[Cu^{2+}] = (10^{7.65})[H^+]^2 \quad (38)$$

or

Malachite: $$[Cu^{2+}] = \frac{(10^{7.08})[H^+]^2}{(P_{CO_2})^{\frac{1}{2}}} \quad (39)$$

or

Azurite: $$[Cu^{2+}] = \frac{(10^{7.08})[H^+]^2}{(P_{CO_2})^{2/3}} \quad (40)$$

The concentrations of the soluble species are then easily calculated:

$$[CuOH^+] = \frac{(10^{-8})[Cu^{2+}]}{[H^+]}, \qquad [Cu(OH)_3^-] = \frac{(10^{-28.3})[Cu^{2+}]}{[H^+]^3}$$

$$[Cu(OH)_4^{2-}] = \frac{(10^{-39.4})[Cu^{2+}]}{[H^+]^4}, \qquad [Cu_2(OH)_2^{2+}] = \frac{(10^{-10.55})[Cu^{2+}]^2}{[H^+]^2}$$

$$[CuCO_3] = (10^{6.77})[Cu^{2+}][CO_3^{2-}], \qquad [Cu(CO_3)_2^{2-}] = (10^{10.01})[Cu^{2+}][CO_3^{2-}]^2$$

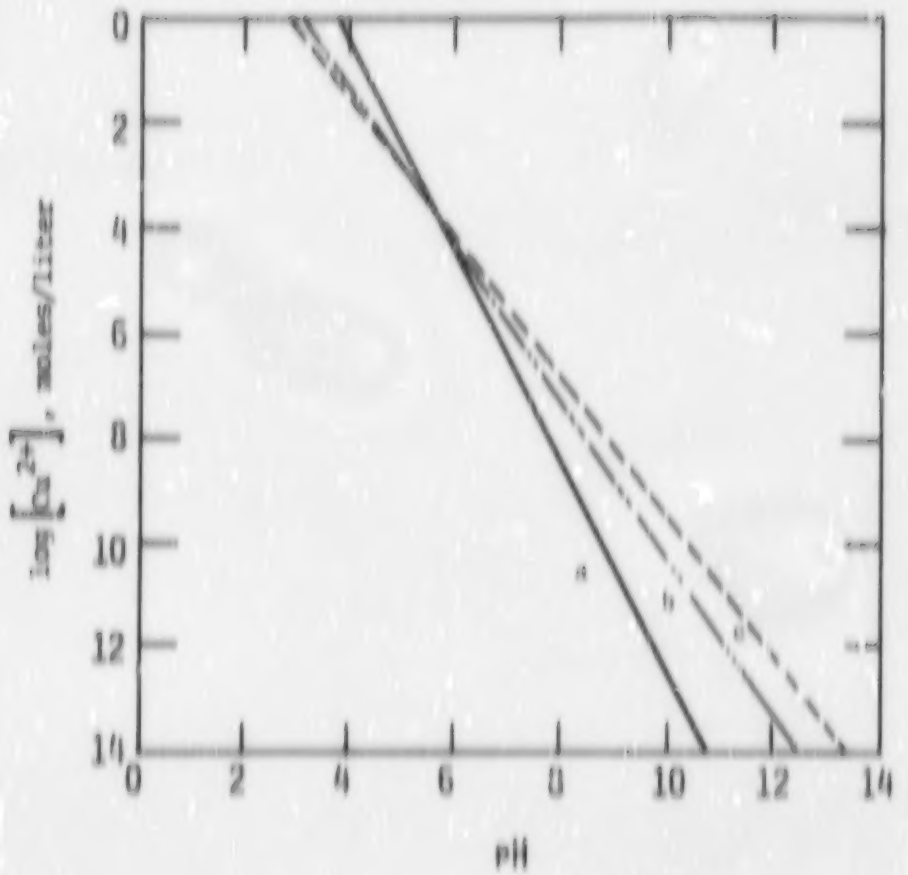


Figure 1. Activity of cupric ion in equilibrium with (a) CuO(s); (b) $Cu_2(OH)_2CO_3$ at bicarbonate ion concentration of 10^{-3} M; and (c) $Cu_3(OH)_2(CO_3)_2$ at bicarbonate ion concentration of 10^{-3} M.

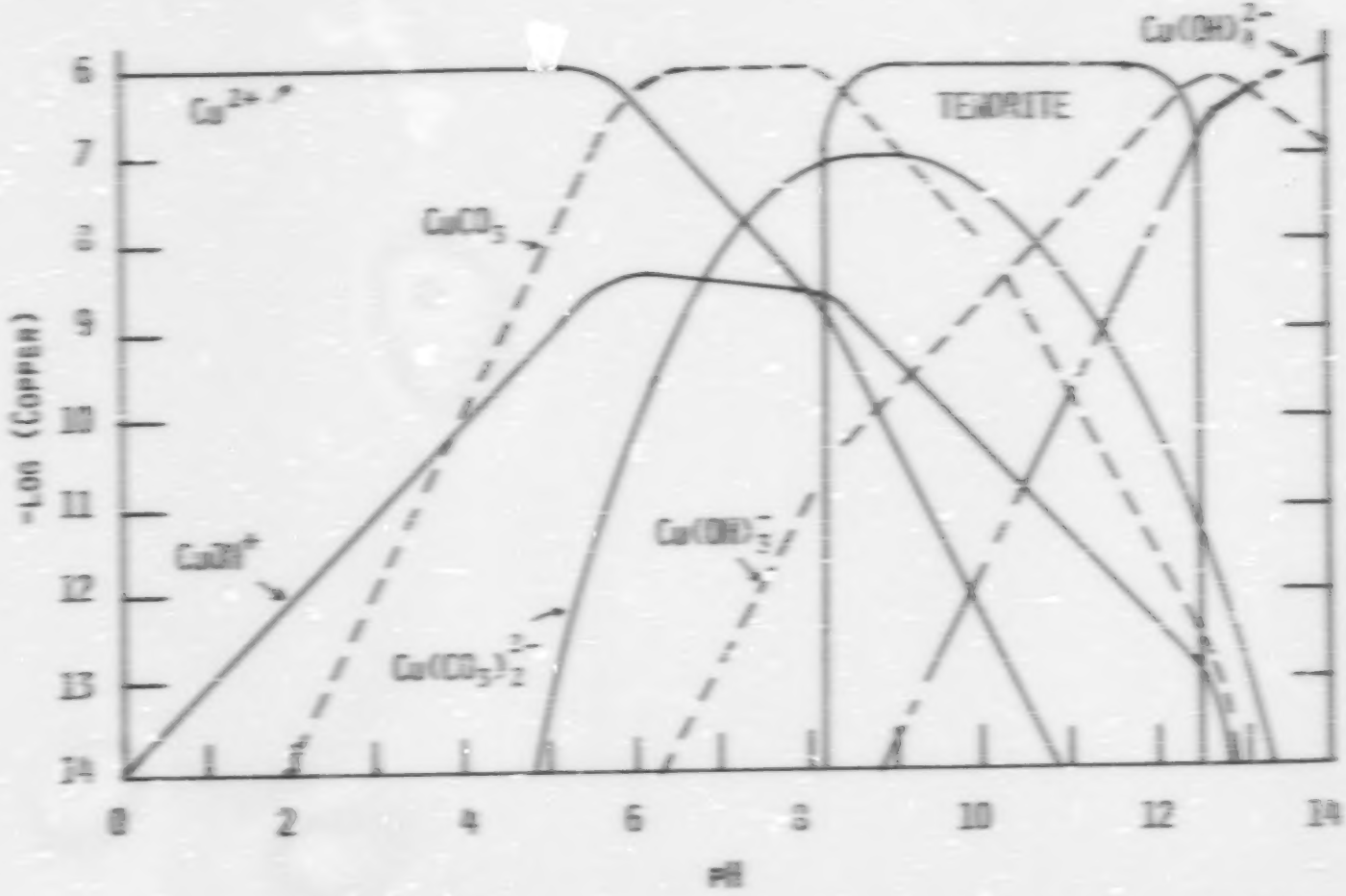


Figure 1. Equilibrium diagram for copper. Total copper concentration equals 10^{-6} m and total carbonate concentration equals 10^{-2} m.

Construction of the Equilibrium Model

A mathematical model can now be constructed that will yield the concentration of each copper species when the total copper concentration, total carbonate concentration, and pH are known.

This model is constructed by the following steps.

1) Assume no solid species are present and calculate Cu^{2+} as explained in case I.
2) Assume a solid species is present and calculate Cu^{2+} as shown in case II.
3) If the value for Cu^{2+} obtained in step (1) is larger than Cu^{2+} calculated in step (2), then precipitation will occur and all calculations should proceed as described in case II.

If the value for Cu^{2+} obtained in step 1 is smaller than Cu^{2+} determined in step 2, then no precipitation occurs and the concentrations of soluble species should be computed as described in case I.

This procedure may be repeated with different pH values and carbonate concentrations to determine the variation in copper species under different chemical conditions. This variation of parameters is a repetitive process, which has been readily accomplished with the use of a computer. Sample computer program output is shown in Appendix A.

A particularly useful method of presenting these data is to plot the pH vs. the log of the concentration of the different copper species, holding the total copper and carbonate concentrations constant. An example of this procedure is shown in Figure 3. With a total copper concentration of 10^{-6} M and a total carbonate concentration of 10^{-2} M, cupric ion predominates from pH 0 to 6, $CuCO_3$ from pH 6 to 8.3, and tenorite from pH 8.3 to 13.5.

In the system described the fraction of the metal present as a given species will be independent of the total metal concentration at any pH and carbonate value as long as the total metal concentration does not become so great as to permit dimeric metallic complexes and providing

that no solid species are present. If metal precipitates form, increasing the total metal concentration will not change the concentration of metal in solution or the species of the metals. Rather, only the quantity of solid will increase. The pH region over which the solid is stable is dependent on the total amount of metal in the system (Figure 4). The range of stability increases as the concentration increases. Since the dominant form of the metal at pH values less than that at which tenorite forms is soluble copper carbonate, it should be expected that the carbonate concentration in addition to the pH would influence the lower limit of tenorite stability. This is shown in Figure 4 by the curved portion of the lower stability boundary. The effect of carbonate is more pronounced as the total copper decreases. At high copper and carbonate concentrations malachite becomes a stable species, but it will transform to tenorite by decreasing either the total copper or carbonate concentration. Again it should be mentioned that equilibrium calculations must be used with caution. The equilibrium constants used in the calculations are generally obtained at higher metal concentrations and ionic strength than are present in the natural environment. Often, predicted stable solid species are not found under experimental conditions. For example, although tenorite is the predicted stable solid, we did not find it to form within 1 week in laboratory systems. Rather, solid copper hydroxide $Cu(OH)_2$ was present. With the same model the predominance of any given copper species as a function of pH and carbonate content can be calculated. This is illustrated in Table I for a total copper concentration of 1×10^{-6} M.

Such equilibrium calculations are considered useful for toxicity studies. They can be used to predict the form of copper under defined experimental conditions. This is particularly relevant since available analytical techniques have not been refined enough to distinguish between metal complexes in solution.

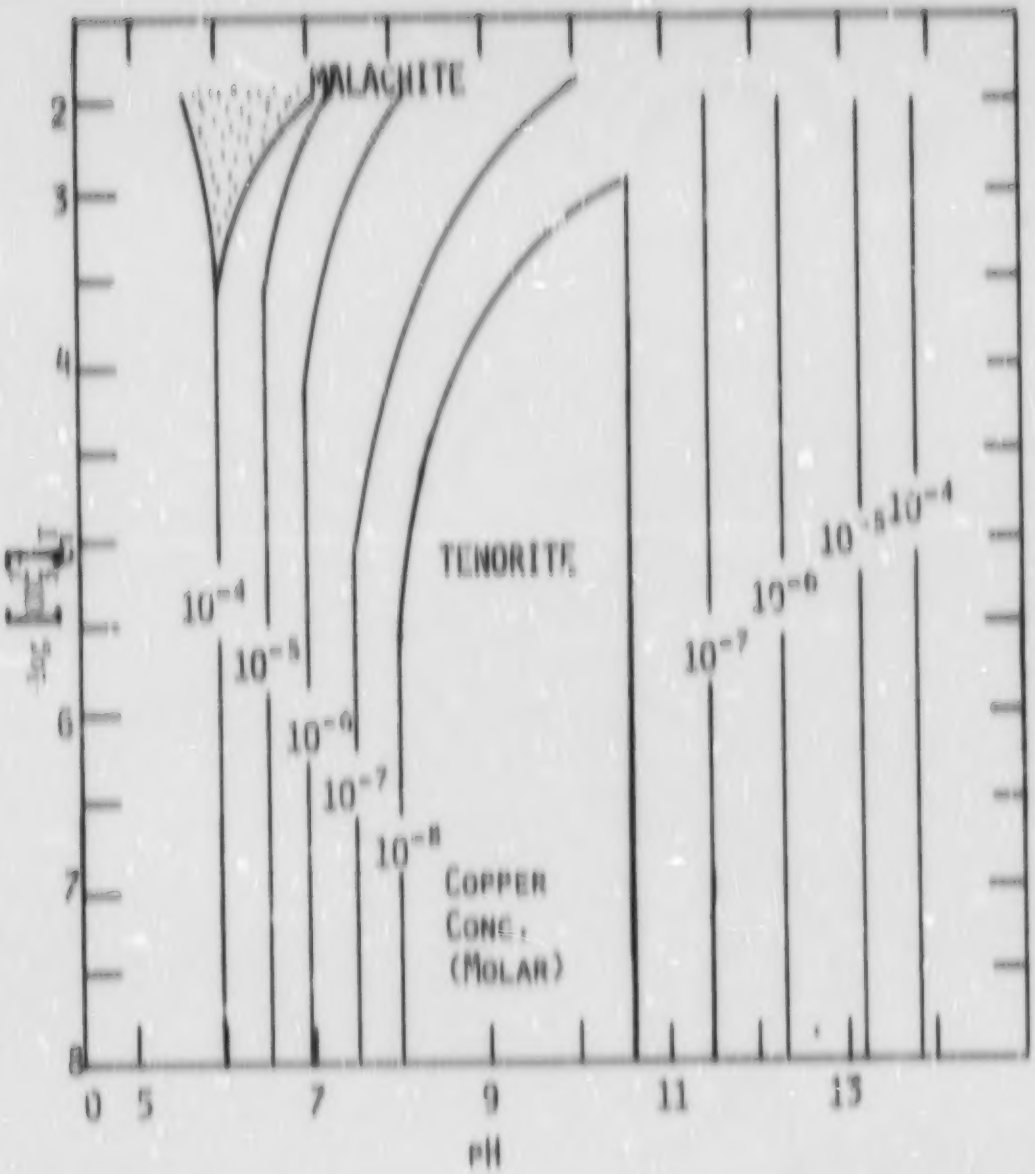


Figure 4. Stability field diagram for tenorite and malachite as a function of pH and total carbonate concentration at various copper concentrations.

Table 1. DISTRIBUTION OF SOLUBLE COPPER AS A FUNCTION OF pH AND TOTAL CARBONATE

Molal/Liter			Soluble copper (%)						
pH	$H_2CO_3^*$	Soluble CO_2	Cu^{++}	$CuCO_3^0$	$Cu(CO_3)_2^{--}$	$CuOH^+$	$Cu(OH)_2^0$	$Cu(OH)_3^-$	$Cu(OH)_4^{--}$
5.5	$10^{-2.5}$	$10^{-2.5}$	99	1	-	-	-	-	-
6.0	$10^{-2.5}$	$10^{-2.5}$	97	3	-	-	-	-	-
6.5	$10^{-2.5}$	$10^{-2.5}$	91	9	-	-	-	-	-
7.0	$10^{-2.5}$	$10^{-2.5}$	77	23	-	-	-	-	-
7.5	$10^{-2.5}$	$10^{-2.5}$	52	48	-	-	-	-	-
8.0	$10^{-2.5}$	$10^{-2.5}$	28	72	-	-	-	-	-
8.5	$10^{-2.5}$	$10^{-2.5}$	12	88	-	-	-	-	-
9.0	$10^{-2.5}$	$10^{-2.5}$	4	96	-	-	-	-	-
9.5	$10^{-2.5}$	$10^{-2.5}$	1	99	-	-	-	-	-
10.0	$10^{-2.5}$	$10^{-2.5}$	-	100	-	-	-	-	-
5.5	$10^{-3.5}$	$10^{-3.5}$	99	1	-	-	-	-	-
6.0	$10^{-3.5}$	$10^{-3.5}$	97	3	-	-	-	-	-
6.5	$10^{-3.5}$	$10^{-3.5}$	91	9	-	-	-	-	-
7.0	$10^{-3.5}$	$10^{-3.5}$	77	23	-	-	-	-	-
7.5	$10^{-3.5}$	$10^{-3.5}$	52	48	-	-	-	-	-
8.0	$10^{-3.5}$	$10^{-3.5}$	28	72	-	-	-	-	-
8.5	$10^{-3.5}$	$10^{-3.5}$	12	88	-	-	-	-	-
9.0	$10^{-3.5}$	$10^{-3.5}$	4	96	-	-	-	-	-
9.5	$10^{-3.5}$	$10^{-3.5}$	1	99	-	-	-	-	-
10.0	$10^{-3.5}$	$10^{-3.5}$	-	100	-	-	-	-	-
5.5	$10^{-4.5}$	$10^{-4.5}$	99	1	-	-	-	-	-
6.0	$10^{-4.5}$	$10^{-4.5}$	97	3	-	-	-	-	-
6.5	$10^{-4.5}$	$10^{-4.5}$	91	9	-	-	-	-	-
7.0	$10^{-4.5}$	$10^{-4.5}$	77	23	-	-	-	-	-
7.5	$10^{-4.5}$	$10^{-4.5}$	52	48	-	-	-	-	-
8.0	$10^{-4.5}$	$10^{-4.5}$	28	72	-	-	-	-	-
8.5	$10^{-4.5}$	$10^{-4.5}$	12	88	-	-	-	-	-
9.0	$10^{-4.5}$	$10^{-4.5}$	4	96	-	-	-	-	-
9.5	$10^{-4.5}$	$10^{-4.5}$	1	99	-	-	-	-	-
10.0	$10^{-4.5}$	$10^{-4.5}$	-	100	-	-	-	-	-

Table 1. (Continued)

pH	Moles/liter		Soluble copper (%)					
	$[CO_3]_T$	Soluble Cu[a]	Cu^{2+}	$CuOH^+$	$Cu(OH)_2^0$	$Cu(OH)_3^-$	$CuCO_3$	$Cu(CO_3)_2^{2-}$
5.5	5×10^{-3}	10^{-6}	93	<1	<1	<1	6	<1
7.0	5×10^{-3}	10^{-6}	7	7	<1	<1	91	<1
8.0	5×10^{-3}	$10^{-6.17}$	<1	1	<1	<1	95	<1
5.5	1×10^{-2}	10^{-6}	89	<1	<1	<1	11	<1
7.0	1×10^{-2}	10^{-6}	4	<1	<1	<1	95	<1
8.0	1×10^{-2}	10^{-6}	<1	<1	<1	<1	92	8

[a] Total copper = $10^{-6.22}$ M

SECTION V

ANALYTICAL CHEMISTRY OF AQUEOUS COPPER

Discussions in this section include a critical review of existing methods for trace copper measurement, with emphasis on their applicability and limitations. More detailed discussions are presented on electroanalytical techniques, specifically anodic stripping voltammetry and potentiometric membrane electrodes. These techniques have been primarily used in this study in conjunction with the bioassay experiments.

GENERAL REQUIREMENTS FOR TRACE METAL ANALYSIS

The principles and procedures of analysis for trace metals, including copper, in the aqueous environment have been described by Hume[51], Maney[52], Morrison[53], and others. The qualities most often noted for an ideal method of trace metal analysis are detection limits, sensitivity, selectivity, the ability to distinguish free from complexed metal, and general applicability for use with natural samples. The analytical method should have low detection limits, sufficient for direct determination of the trace metal without pretreatment or preconcentration steps. Copper, for instance, occurs at levels of 10^{-7} to 10^{-9} M in natural waters, and an appropriate method should be able to reach this level of detection with reliability. Selectivity implies the capability to distinguish a signal that is due strictly to the trace metal in question and not to the presence of any interfering ion that may be present in the test solution.

Ideally speaking, the analytical procedure should also be capable of defining the metal species under investigation. Lack of information on the speciation of trace metals has been a main obstacle to the elucidation of their role in natural waters. Information on the type of species present (e.g., type of complexes and their stability and rates of complex formation) is a prerequisite to a better understanding of the distribution and function of trace metals in aquatic environments.

Copper does not exist in simple ionic form in natural waters. The work of Corcoran and Alexander[9], Slowey and Hood[10], and Foster and Morris[54] has led them to believe that as much as 50 percent of the copper present in sea water is in the form of organic complexes. Later work by Barber and Ryther[24] and Johnston[22] has shown that the organic complexes of copper can be correlated to the biological activity in sea water.

The applicability of a method sums up a variety of factors of practical consideration. The reliability, reproducibility, and precision of the method in question all fall under the heading of applicability. Other fundamental considerations are (1) the portability and ruggedness of equipment for field operations, (2) the time required for a single analysis, (3) the simplicity of the operation, and finally (4) economy, space, and additional equipment requirements.

Available Analytical Methods for Trace Copper Analysis

The analyst can select among a number of analytical procedures with different performance characteristics[55-60]. Various trace analysis techniques and their general sensitivity ranges are shown in Table 2. The following discussion will center on the applicability of some of the more common techniques for copper and includes spectrophotometric, radiochemical, and electroanalytical methods of trace copper determination.

Table 2. SENSITIVITIES OF INSTRUMENTAL METHODS FOR MONITORING METALS IN WATER[a]

Method	Sensitivity, ppb (ug/l)
Polarography	100 - 1,000
Molecular Absorption	100 - 1,000
Atomic Absorption (Flame)	100 - 1,000
Atomic Emission	100 - 1,000
Square Wave Polarography	10 - 100
Sweep Voltammetry	10 - 100
Emission Spectroscopy (Arc)	10 - 100
X-Ray Fluorescence	10 - 100
Pulse Polarography	1 - 10
Molecular Fluorescence	1 - 10
Spark Source Mass Spectrometry	1 - 10
Anodic Stripping	0.1 - 10
Neutron Activation	0.1 - 10
Atomic Absorption (Flameless)	0.1 - 10
Chemiluminescence	0.1 - 10
Emission Spectroscopy (Plasma)	0.1 - 10

[a]For any method the sensitivity will vary according to the particular metal.

Optical Techniques -- The optical techniques most commonly used in trace copper determination include atomic absorption spectrophotometry and colorimetry. In recent years atomic absorption has become one of the most popular methods of analysis for copper in natural and waste waters because of its selectivity, speed, and relative simplicity of analysis. The technique requires as little as a 5- to 10-ml sample to perform analysis, and sensitivities in the region of microgram of copper per liter have been achieved with normal flame absorption techniques (55). Non-flame techniques can improve on this sensitivity, but are more costly and are severely lacking in precision. The main limitations of both flame and flameless atomic absorption techniques are: (a) metal species cannot be determined; (b) the technique is strictly a laboratory procedure and cannot be used in the field; and (c) cost per analysis is high in comparison to electroanalytical procedures.

Colorimetry, in contrast to atomic absorption, is simple and inexpensive. The American Public Health Association et al. (57) prescribe three colorimetric methods for use in copper determination, and these methods are capable of achieving detection limits near 20 ppb depending on the experimental conditions and the presence of interfering ions.

1. The cuprethol method achieves its maximum sensitivity with 100-ml nessler tubes for visual comparison, but mercurous, nickel, silver, bismuth, and cobalt ions can act as significant interferences.
2. The bathocuproine method is just as sensitive as the cuprethol method, and no ions interfere significantly when in concentrations below the parts-per-million range.
3. The most specific reagent for copper, neocuproine, is most commonly used for analysis of aqueous copper.

The main limitation with colorimetric procedures is lack of selectivity and the effect of interfering ions. In addition, colorimetric procedures require a number of preparatory steps as well as a separation or concentration step. Colorimetric procedures are also incapable of metal speciation since complex forms of copper may interact slowly or not at all with the color-developing compound.

Radiochemical Techniques -- Neutron activation analysis is a very sensitive analytical procedure with detection limits in the parts-per-trillion (ppt) range[58]. Hence, no preconcentration step is necessary. Nevertheless the matrix effect can be quite significant and may result in suppressing the signals. Beyond matrix effects, the most serious limitations of activation analysis are its inability to distinguish species of trace metals and the elaborate, sophisticated, and expensive equipment required for operation.

Miscellaneous Techniques -- Several techniques that are not easily categorized have been used in recent years for trace analysis. Mass spectroscopy is a very sensitive technique for trace copper analysis, but extensive sample preparation is required and the overall method is time consuming and expensive and requires skilled analysts[59].

Electron spin resonance has also been used for the analysis of copper [60].

Electroanalytical Techniques -- Among the various electroanalytical techniques particularly applicable for the analysis of copper in aqueous samples are (a) potentiometric membrane electrodes, (b) conventional and pulse polarography, and (c) anodic stripping voltammetry. These techniques vary in their limit of detection from 10^{-5} M for conventional polarography to 10^{-10} M for anodic stripping voltammetry.

Perhaps the unique advantage of electroanalytical techniques lies in their ability to distinguish between free and complexed metallic species. Additional advantages of these methods are their capability of in situ

measurement, their high selectivity, and their easy adaptability for field operations. Furthermore, electroanalytical measurement of metals in aqueous samples is considerably less expensive than atomic absorption spectrophotometry. The comparison is based on capital cost of equipment and cost per analysis including manpower requirements.

The oldest of the above-mentioned techniques is conventional polarography, with a detection limit for copper of about 10^{-5} M, a range of practical operation of 10^{-3} to 10^{-5} M, and a precision of 1 - 3 percent. If preconcentration steps are used, the sensitivity can be lowered to about 10^{-7} M.

Cathode-ray polarography or oscillographic polarography has been used for analysis of natural water and wastewaters with a detection limit of 10^{-7} M[61,62]. This technique involves the use of a cathode-ray oscilloscope to measure the current-potential curves of applied (saw-tooth) rapid potential sweeps during the lifetime of a single mercury drop. Multiple techniques are also applicable. The peak current in the resulting polarogram is related to the concentration of the electroactive species for a reversible reaction. Oscillographic polarography has the advantages of (1) relatively high sensitivity, (2) high resolution, and (3) rapidity of analysis. Copper can be determined at the 50 ppb level in natural waters by this technique[63].

The basic polarographic techniques are further modified in pulse and differential pulse polarography, both of which may be used to increase the sensitivity and minimize the effects of interferences[64]. Pulse polarography has the advantage of extending the sensitivity of determination to approximately 10^{-8} M. The pulse technique is based on the application of short potential pulses of 50 msec on either a constant or a gradually increasing background voltage. Following application of the pulse, current measurements are usually done after the spike of charging current has decayed. The limiting current in pulse polarography is larger than in classical polarography. In derivative pulse polarography, the voltage pulse is superimposed upon a slowly changing

potential (about 1 mV per sec) and the difference in current between successive drops is recorded versus the potential.

Differential pulse polarographic techniques are based on measuring the current twice during the lifetime of each mercury drop and recording the difference[64]. Current peak-shaped curves are obtained where the peak height is proportional to the metal concentration. This technique is 100 - 1,000 times more sensitive than classical polarography, and 10 times more sensitive than nondifferential pulse techniques.

Anodic stripping voltammetry (ASV), sometimes referred to as inverse polarography, is a simple yet extremely useful electroanalytical technique for trace metal analysis. It offers a detection limit of as low as 10^{-8} to 10^{-10} M for copper, which is well within the range of naturally occurring levels. Moreover, ASV, along with other electrochemical methods, has the ability to distinguish free from complexed metal. The method is highly specific for copper and, even at very low concentrations, the precision is near 5 percent. The equipment required is relatively inexpensive, readily transported, and has been used in the field.

The application of ASV to environmental studies has until recently received little mention in the literature. Matson and coworkers have been the forerunners of bringing attention to this useful technique. Their work involves studies on the Great Lakes[18,65], arctic lakes[66], and sewage effluents[67]. Evidence for the existence of trace metal-organic complexes is clearly presented in these works; however, the techniques utilized for the characterization of these complexes generally involved only acid digestion and acid titration procedures. The only other available study on the application of ASV to the characterization of trace metal complexation is that of Zirino and Healy[68]. Their study of the analysis of zinc in sea water by ASV included the observation that the peak current associated with zinc decreased as the pH increased. This phenomenon was explained on the basis of the increased formation of $ZnCO_3$ or $Zn(OH)_2$ with increasing pH.

Other environmental studies with ASV have been primarily concerned with the quantitative determination of trace metals in aquatic systems. Peterson, Brant, and Nancy[69] recently used the composite mercury-graphite electrode (CMGE) in the study of the distribution of copper and lead in a municipal water system. This study included seasonal, daily, and hourly variations in the concentrations of these metals at various points along the distribution route of municipal water. Variation in the concentrations of the metals was related to residence times in the pipes as well as variations in the original source of the water and in the rate of consumption. Anodic stripping voltammetry was chosen as the primary analytical technique for the characterization of copper in the bioassay study. In the following section a detailed discussion of the experimental procedures and methods of metal speciation is presented.

ANODIC STRIPPING VOLTAMMETRY

Introduction

Anodic stripping voltammetry (ASV) is a combination of (a) a concentration step (pre-electrolysis, cathodic deposition, or plating) in which the metal ion (or ions) of interest is reduced by controlled potential electrolysis, either by deposition as a metal onto a solid microelectrode or by formation of an amalgam with the mercury of a mercury drop or mercury film electrode and (b) a following step consisting of an anodic dissolution process (reverse electrolysis or stripping) in which the metal is oxidized and returned to the solution. As with other electrochemical techniques ASV is a nondestructive method of analysis.

The concentration step is carried out for a definite time under reproducible conditions such that the concentration onto the electrode is quantitative (total plating) or such that a reproducible fraction of the desired component is deposited from solution (partial plating). By controlling the potential during this process, more easily electrolysed constituents may be separated. The concentration step is thus performed

by applying to the electrodes a potential which is cathodic of the polarographic halfwave potential by some 0.3 to 0.4 volts (where the current has its limiting value) and maintaining this potential for a given period of time under reproducible conditions of stirring, type and area of microelectrode, and composition of medium.

After a short "rest period" to allow the solution to become quiescent, the stripping process is initiated. The stripping step is performed by a voltammetric scanning procedure, which produces a response proportional to the amount of material deposited. The resulting stripping "voltammogram" produces peaks whose heights are generally proportional to the concentrations of the corresponding electroactive metal ions and whose potentials are a qualitative indication of the nature of the species present in solution. On this basis, the important characteristics of the peak are its height (peak current, i_p, in microamperes), its width at $\frac{1}{2}i_p$ ($W_{\frac{1}{2}}$, in volts or millivolts), and its potential (E_p, in volts). These characteristics are affected by the type of microelectrode used and by the rate of voltage change (sweep rate, v, in mV/sec) used in the stripping process.

Anodic stripping voltammetry, which is also known as linear potential sweep stripping chronocamperometry, stripping analysis, anodic amalgam voltammetry, and inverse voltammetry, has been successfully applied to the analysis of Ag, Au, Ba, Bi, Cd, Ga, Ge, Hg, In, K, Mn, Ni, Pb, Pt, Sb, Sn, Tl, and $Zn^{(70)}$, in addition to the analysis of Cu.

Theory

In the plating step, metal deposition follows a first-order rate expression

$$Q_t = Q_0 (1 - e^{-kt})$$

where Q_t = number of coulombs equivalent to metal deposited at time t (coulombs)

Q_0 = number of coulombs equivalent to total amount of metal in test solution (coulombs)

t = time of electrolysis (seconds)

k = rate constant defined by the expression (sec^{-1})

$$k = DA/V\delta \qquad (41)$$

where D = diffusion constant of the electroactive species in solution (cm^2/sec)

A = test electrode surface area (cm^2)

V = volume of the solution (cm^3)

δ = effective thickness of the diffusion layer (cm)

It is essential to conduct the plating step at conditions of constant stirring, temperature, cell geometry, and supporting electrolyte.

The stripping step is normally conducted in a quiescent solution. The voltammetric technique normally employed during the stripping phase is potential sweep chronoamperometry (application of an anodic linear potential sweep); however, other voltammetric procedures may also be applied. Of those which have been attempted, square wave polarography[71], radio frequency techniques[72], differential polarography[73], and pulse polarography[74] have proven successful.

The working electrode employed in this study was the thin-film mercury graphite electrode[75]. The current-potential equations for the anodic stripping curves with mercury thin-film electrodes were derived and verified by Nae and Toni[76]. Their derivations result in the following equations:

$$i_p = nFAlC_R^0 v\phi/e \tag{42}$$

$$E_p = E_0 + \frac{2.3}{\phi} \log \frac{\delta l v \phi}{D_0} \tag{43}$$

where
- n = number of electrons in the electrode reaction
- F = Faraday
- A = surface area of the electrode
- l = thickness of the mercury film
- C_R^0 = concentration of reduced metal in the electrode
- v = rate of potential change
- ϕ = nF/RT
- δ = thickness of the unstirred layer of solution adjacent to the electrode
- D_0 = diffusion coefficient of the oxidized species in solution

These equations predict that i_p is a linear function of v, and E_p is a linear function of $\log v$. The equations have been experimentally verified and have been found to be valid at low sweep rates. Large values of v can cause departures from the equations because of a breakdown in the assumptions that (a) δ is a constant from one sweep rate to the next and (b) the diffusion rate of deposited metal out of the mercury film is extremely rapid compared to the sweep rate. The effect may be significant for i_p, but is only slight for E_p.

Regardless of which type of electrode is used in ASV, the analytical results obtained from the stripping curve are always dependent in some way on the sweep rate and on some property of the geometry of the elec-

trode. These factors profoundly influence the peak characteristics, namely, the peak current, the peak potential, and the peak width.

Diagnostic Criteria

Trace metals in the aquatic environment are frequently found in the parts-per-billion range or lower (10^{-8} - 10^{-10} M). With most analytical procedures such low levels necessitate a preconcentration step before the actual analysis. Anodic stripping voltammetry with the composite mercury-graphite electrode (CMGE), however, has a great enough sensitivity to permit direct determinations of metal ions at these concentration levels. In addition, it is a nondestructive process which permits repeated determinations on the same sample. Therefore, ASV is a technique highly suited for environmental samples.

Additionally, ASV also offers the unique opportunity to study trace metal complexation at the environmentally significant levels. This is based on the following diagnostic procedures for the differentiation of free and complexed metals and for the characterization of metal complexes: (a) acid digestions; (b) variations of ASV parameters, namely, sweep rate, plating time, and plating potential; (c) titrations of natural water samples with acid, metals which cannot be determined by ASV, metals which can be determined by ASV, and titrations of the trace metals in water samples with organic ligands. Total metal content can be determined by digesting the sample before ASV analysis, and filtration separates dissolved from particulate fractions on the basis of particle size. The analytical scheme is illustrated in Figure 5.

In a discussion of the types of complex systems that might be encountered in bioassay waters, there are two broad classifications with several subclassifications depending on the types of ligands present:

Case I. Excess trace metal (M)

a. (M) and (ML)

b. (M) and (M_2L)

c. (M) and (ML) and (M_2L)

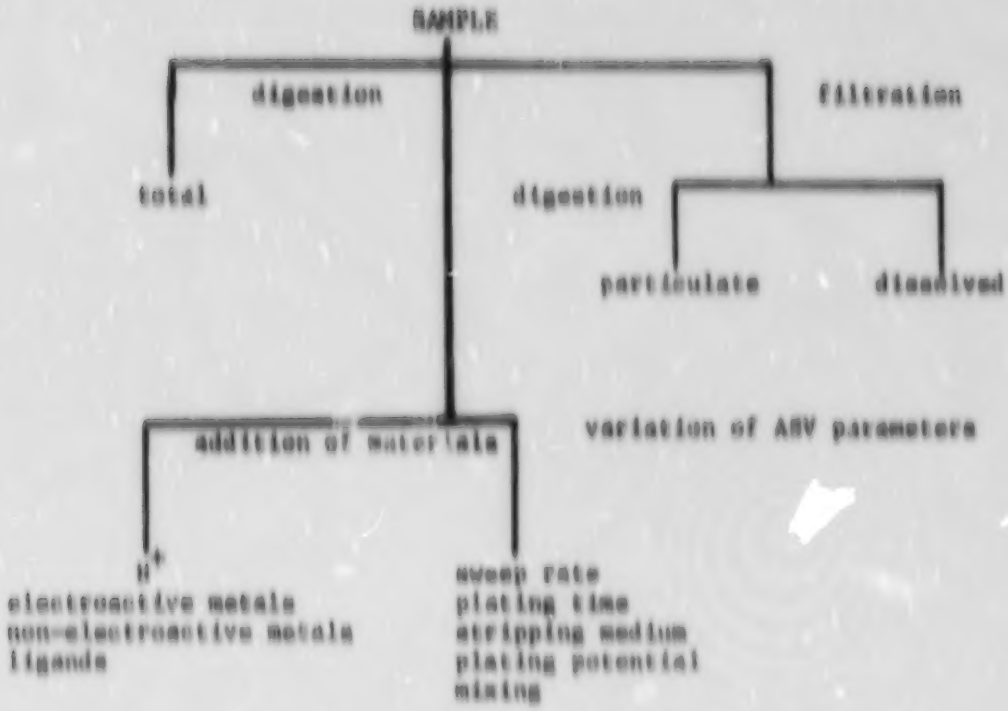


Figure 5. Analytical scheme for anodic stripping voltammetric determination of metals in natural waters.

Case II: Excess ligand, (LL) or (ML)

a. (LL) and (MLL) and (MNL)

b. (LL) and (ML) and (MNL) where the nonlabile complex (MNL) is the stronger complex

c. (ML) and (MLL) and (MNL)

d. (LL) and (ML) and (MNL) where the labile complex (MLL) is the stronger complex

In this classification M stands for the metal, LL is a labile ligand which forms complexes whose rate of dissociation is faster than the rate of plating, and ML is a nonlabile ligand which forms complexes whose rate of dissociation is slower than the rate of plating. Note that the definition of labile and nonlabile ligands is an operational definition based on the dissociation kinetics of their respective complexes. This should be differentiated from the definition of weak and strong complexes which have thermodynamic significance based on stability constants. Note also that the above classification assumes a water sample containing a mixture of available ligands.

Based on the above limitations of the terms labile and nonlabile, it can be concluded that i_p measurements can differentiate between free metals and labile metal complexes on one hand and nonlabile metal complexes on the other hand. A further differentiation between labile and nonlabile metal complexes is possible from data obtained during metal-organic ligand titrations. E_p measurement can generally distinguish between free metals and complexed metals, both labile and nonlabile. The stability constants can easily by calculated from E_p measurements. Furthermore, the technique is suitable for assessing rates of metal complexation. This is illustrated in Figure 6.

Results from the titrations of metal by the addition of organic ligands provide evidence for metal-organic complexation; however, this technique, in most cases, cannot be used to characterise the type of metal-organic interaction. On the other hand, metal-organic titration in which the metal is the titrant is a useful diagnostic criterion for the characterisation of both labile and nonlabile ligand-metal interactions[77].

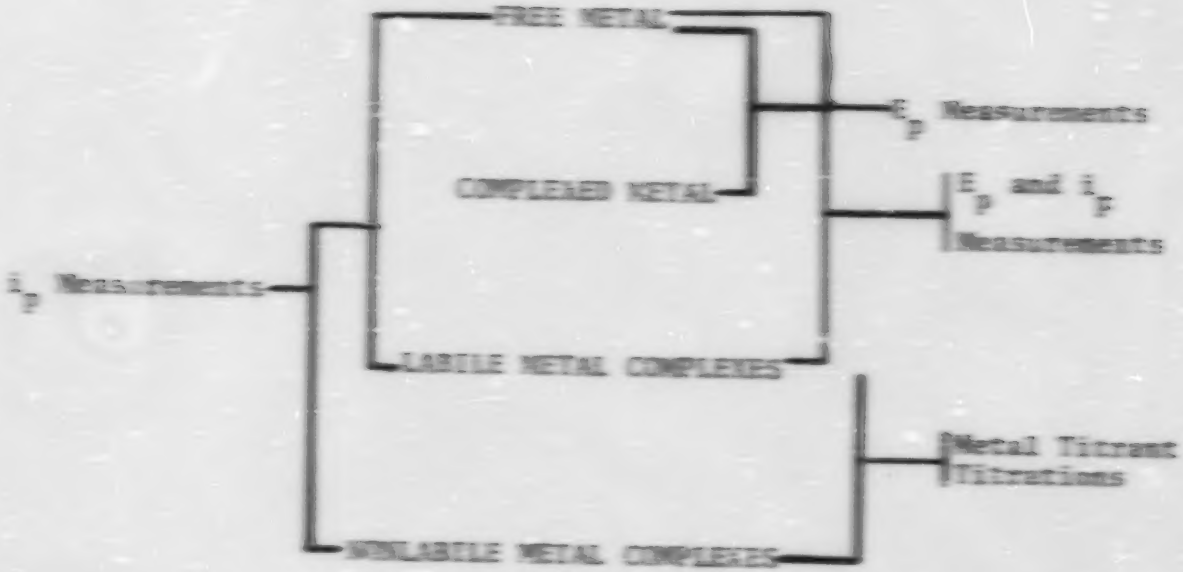


Figure 6. Anodic stripping voltammetric characterization of metallic species.

Experimental

Heath polarography system, Model EUW-401, (Heath Company, Benton Harbor, Michigan) and a chopper stabilizer, Model EUA-19-4, were used in this work. A polarographic module interface unit, Model 1014, (Environmental Sciences Associates, Inc., Newtonville, Massachusetts) in conjunction with a Heath operational amplifier, Model EUW-19A, was used to provide the necessary voltammetric deposition and stripping modes for the simultaneous operation of four cells. Voltammograms were recorded on a Honeywell Electronik 194 strip chart recorder (Honeywell, Inc., Fort Washington, Pennsylvania).

Measurements were performed in 100-ml fused quartz beakers, and the electrodes were inserted into the cells through rubber stoppers. The cell and electrode arrangement is shown in Figure 7.

The reference and counter electrodes were both separated from the solution by 4-mm-diameter Vycor plugs. These plugs were attached to the electrode proper (composed of 4-inch lengths of 4-mm-diameter Pyrex tubing) by means of sleeves of Teflon tubing.

The counter electrode was filled with supporting electrolyte. Electrical contact was made by means of a platinum wire inserted in the tube. Contact to the solution in the cell was made through the porous Vycor plug. A saturated mercury-mercurous sulfate reference electrode was used and constructed as described by Lingane[78]. The test electrode was a composite mercury-graphite electrode (CMGE). The electrode surface area was 3.2 cm^2. Detailed discussions on the preparation and properties of the CMGE are found elsewhere[75,79].

A nitrogen-carbon dioxide gas mixture was used for deaeration of the test solution. The pH was controlled by using appropriate partial pressures of carbon dioxide.

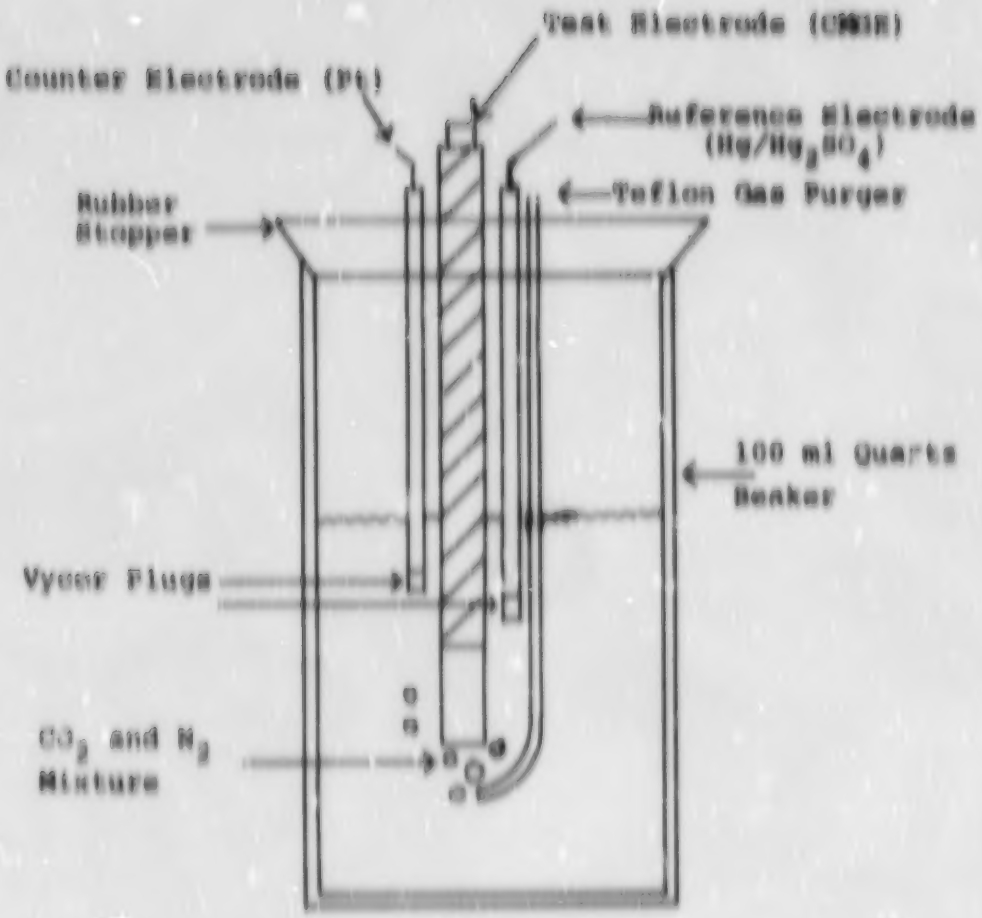


Figure 7. Cell and electrode arrangement for anodic stripping voltammetry.

STUDY OF COMPETITIVE METAL INTERACTIONS

Water samples containing 5 ppm humic acid were titrated with copper in the presence and absence of 40 ppm calcium. The titration curves shown in Figure 8 illustrate the competitive interaction between copper and calcium for humic acid ligands[77]. The lack of peak current signal at low Cu concentrations was believed to be a result of the formation of strong nonlabile complexes. At high pH values the capacity for nonlabile metal complex formation is greater than at low pH values. After the nonlabile ligand has been saturated, further addition of copper to the test solution is accompanied by an increase in peak current signals. The presence of calcium exhibits little or no effect on copper-humic acid interactions. Thus, copper competes favorably with calcium for the formation of a nonlabile complex with humic acid.

For comparative purposes Figure 9 shows the same titration curves with cadmium. These titration curves are clearly linear, indicating predominantly cadmium-humic acid labile interactions. Furthermore, the presence of calcium causes a release of cadmium from its complexes and allows for a more rapid interaction between the trace metal and the test electrode. This is evidenced by the increased peak currents obtained in the presence of calcium.

Examples shown in Figures 8 and 9 represent two different heavy metal-organic interactions. Copper forms relatively stable, nonlabile complexes with humic acid, but cadmium seems to form more labile complexes. Calcium competes favorably with cadmium, and not with copper for complex formation with humic acid. Additionally, humic acid represents a mixture of labile and nonlabile ligand characteristics. Because in the case of cadmium, the addition of calcium causes an increase in peak currents, we believe that the cadmium-humic acid interaction is not exclusively labile. A summary of metal-humic acid-calcium interactions for a number of trace metals is given in Table 3.

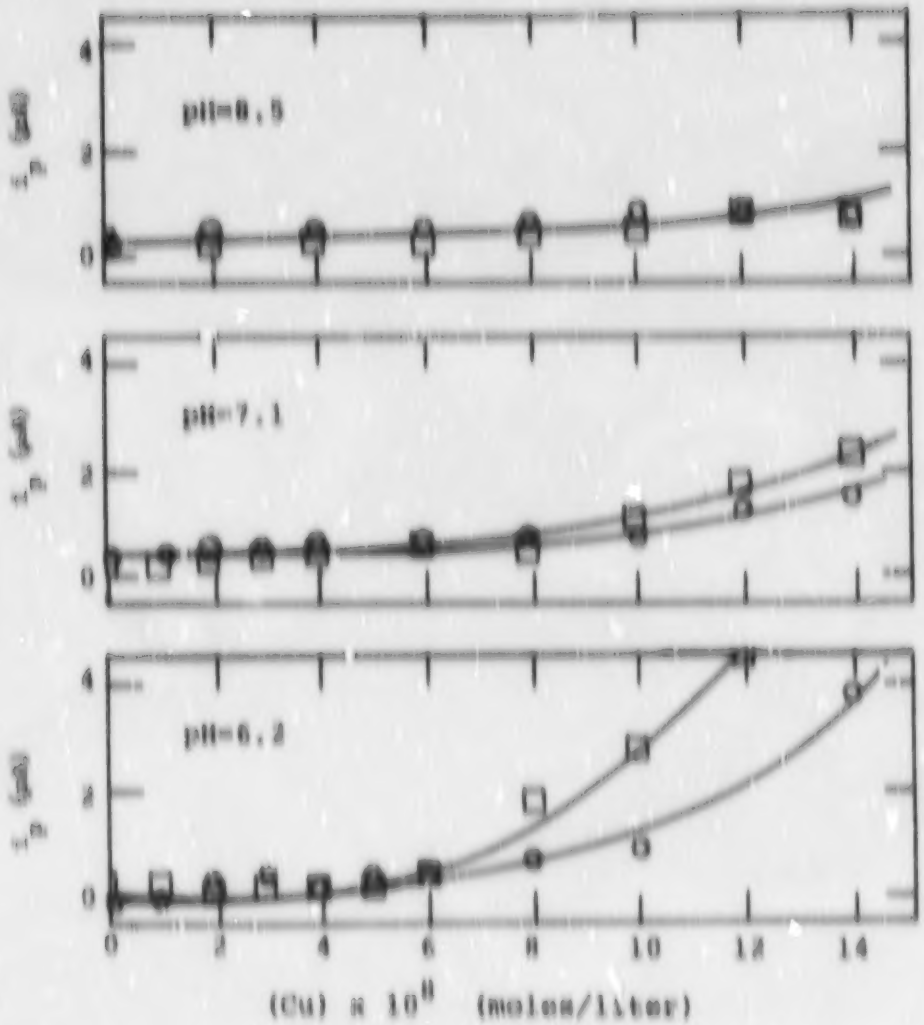


Figure 8. The effect of calcium and pH on the variation of the peak current during the titration of 5 µg/ml humic acid with copper.

● Calcium absent
■ Calcium present

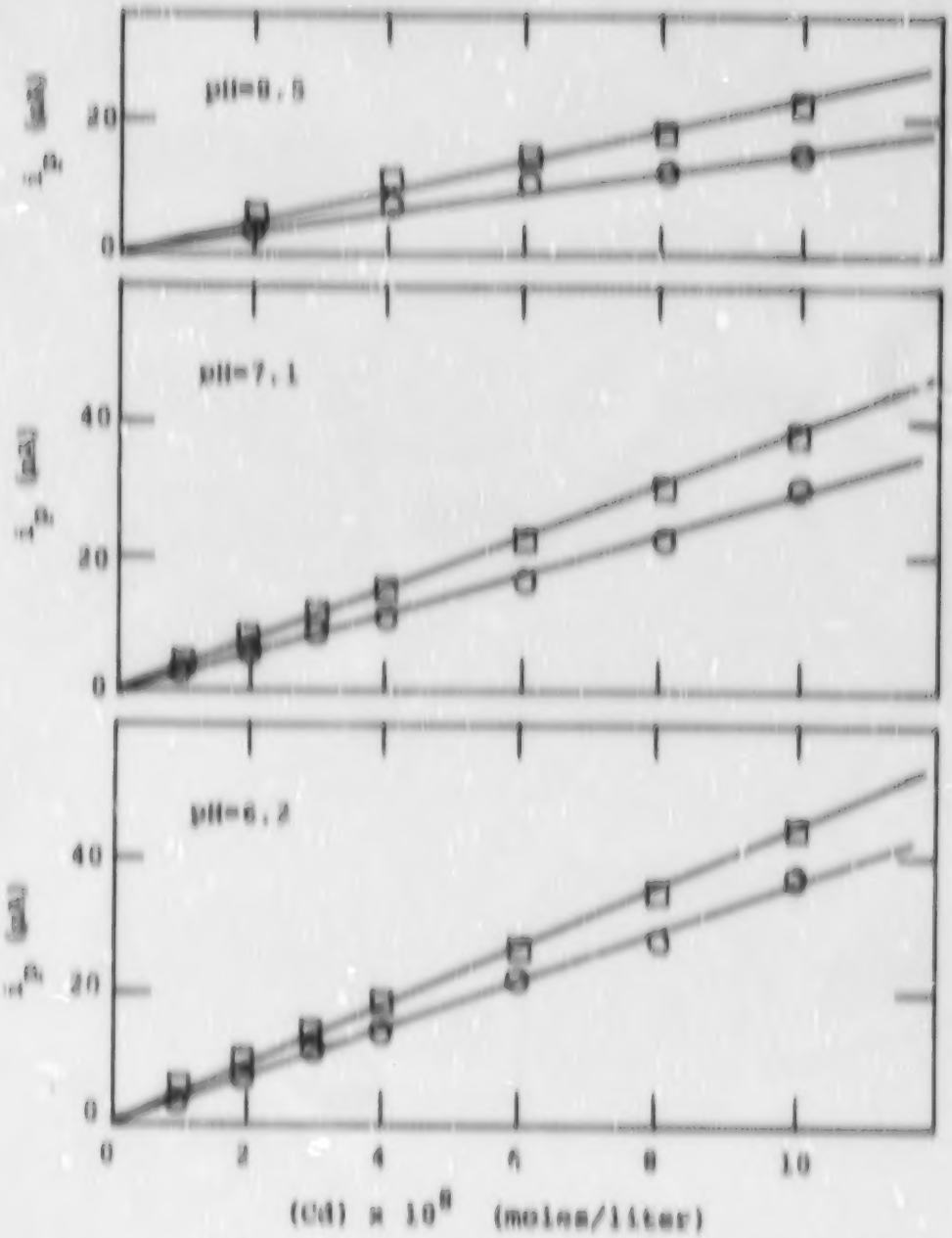


Figure 9. The effect of calcium and pH on the variation of the peak current during the titration of 5 ug/ml humic acid with cadmium.

● Calcium absent
■ Calcium present

Table 3.

SUMMARY OF TRACE METAL-HUMIC ACID-CALCIUM INTERACTIONS AS OBSERVED DURING TITRATIONS WITH METAL IONS

Metal	Nature of Interactions
Bismuth	Nonlabile interactions becoming increasingly labile with increasing pH; successful calcium competition for labile ligands
Cadmium	Labile interactions; successful calcium competition
Copper	Strong nonlabile interactions increasing with increasing pH; successful calcium competition for labile ligands
Indium	Nonlabile interactions; successful calcium competition for labile ligands
Lead	Increasing lability with increasing pH; successful calcium competition increasing with increasing pH
Thallium	Labile interactions; successful calcium competition

POTENTIOMETRIC MEMBRANE ELECTRODES

An Orion cupric ion selective electrode was used in this study to measure Cu^{2+} concentrations. Absolute potentiometric measurements followed a Nernstian relationship. The detection limit for this electrode system was $10^{-5.5}$ M. Pertinent information on metal speciation can be achieved from potentiometric titrations.

The formation of the soluble complex species $CuCO_3$ has been studied by Scaife[80], Stirsan[81], and Stiff[82]. This complex is found in natural waters at pH 6.5 to 8.0; its concentration is governed by the concentration of carbonate.

The equilibrium constants for the reaction between Cu^{2+} and HCO_3^- were studied. A modified procedure from that described by Stiff[82] was used. This included rigorous control of pH and total carbonate. Based upon the techniques reported by Stiff[82], but in which both copper activity and pH were monitored, we have determined the equilibrium constant for the reaction of cupric and bicarbonate ions to form $CuCO_3$. Three concentrations of sodium bicarbonate (1.0×10^{-3}, 2×10^{-3}, and 5×10^{-3} M) and three concentrations of copper (1.58×10^{-6}, 7.9×10^{-6}, and 1.58×10^{-5} M) were used.

It was assumed that the following two equilibrium relationships govern the copper-bicarbonate system:

$$Cu^{2+} + HCO_3^- \rightleftharpoons CuHCO_3^+, \quad K_1 = [CuHCO_3^+]/[Cu^{2+}][HCO_3^-] \qquad (44)$$

$$Cu^{2+} + HCO_3^- \rightleftharpoons CuCO_3 + H^+, \quad K_2 = [CuCO_3][H^+]/[Cu^{2+}][HCO_3^-] \qquad (45)$$

Under these conditions, the total copper $[Cu_t]$ is approximated by the following equation:

$$[Cu_t] = [Cu^{2+}] + [CuCO_3] + [CuHCO_3^+], \text{ or}$$

$$[CuHCO_3^+] + [CuCO_3] = [Cu_t] - [Cu^{2+}], \tag{46}$$

which by substitution gives,

$$([Cu_t] - [Cu^{2+}])/[Cu^{2+}][HCO_3^-] = K_1 + K_2/[H^+]. \tag{47}$$

The values of $([Cu_t] - [Cu^{2+}])/[Cu^{2+}][HCO_3^-]$ when plotted against the corresponding values of $1/[H^+]$ would be linear if equations (45) and (46) govern this system. The slope of the line will be equal to K_2, and the intercept of the $([Cu_t] - [Cu^{2+}])/[Cu^{2+}][HCO_3^-]$ axis will be equal to K_1.

The results obtained with different bicarbonate solutions are shown in Figure 10. The graphs have the following features: (a) The intercepts on the $([Cu_t] - [Cu^{2+}])/[Cu^{2+}][HCO_3^-]$ axis are all very close to the origin showing that K_1 is close to zero and that $CuHCO_3^+$ is a virtually non-existent species. (b) Below a pH value of 8.0 in 5.0×10^{-3} M bicarbonate, pH 7.6 in 2.0×10^{-3} M bicarbonate, and pH 7.3 in 1.0×10^{-3} M bicarbonate equations (44) and (45) do represent the system and since K_1 is nearly zero $[Cu_t] = [Cu^{2+}] = [CuCO_3]$. (c) At pH values greater than those indicated the plots are nonlinear, indicating the formation of a complex or complexes in addition to $CuCO_3$. The values of K_2 were computed from the slopes of the linear portions of the graphs. The values of K_2 vary with varying total ionic strength. The thermodynamic equilibrium constant K_2' was calculated from the equation:

$$K_2' = (K_2)\ (a_{CuCO_3})\ (a_{H^+})/(a_{Cu^{2+}})\ (a_{HCO_3^-}). \tag{48}$$

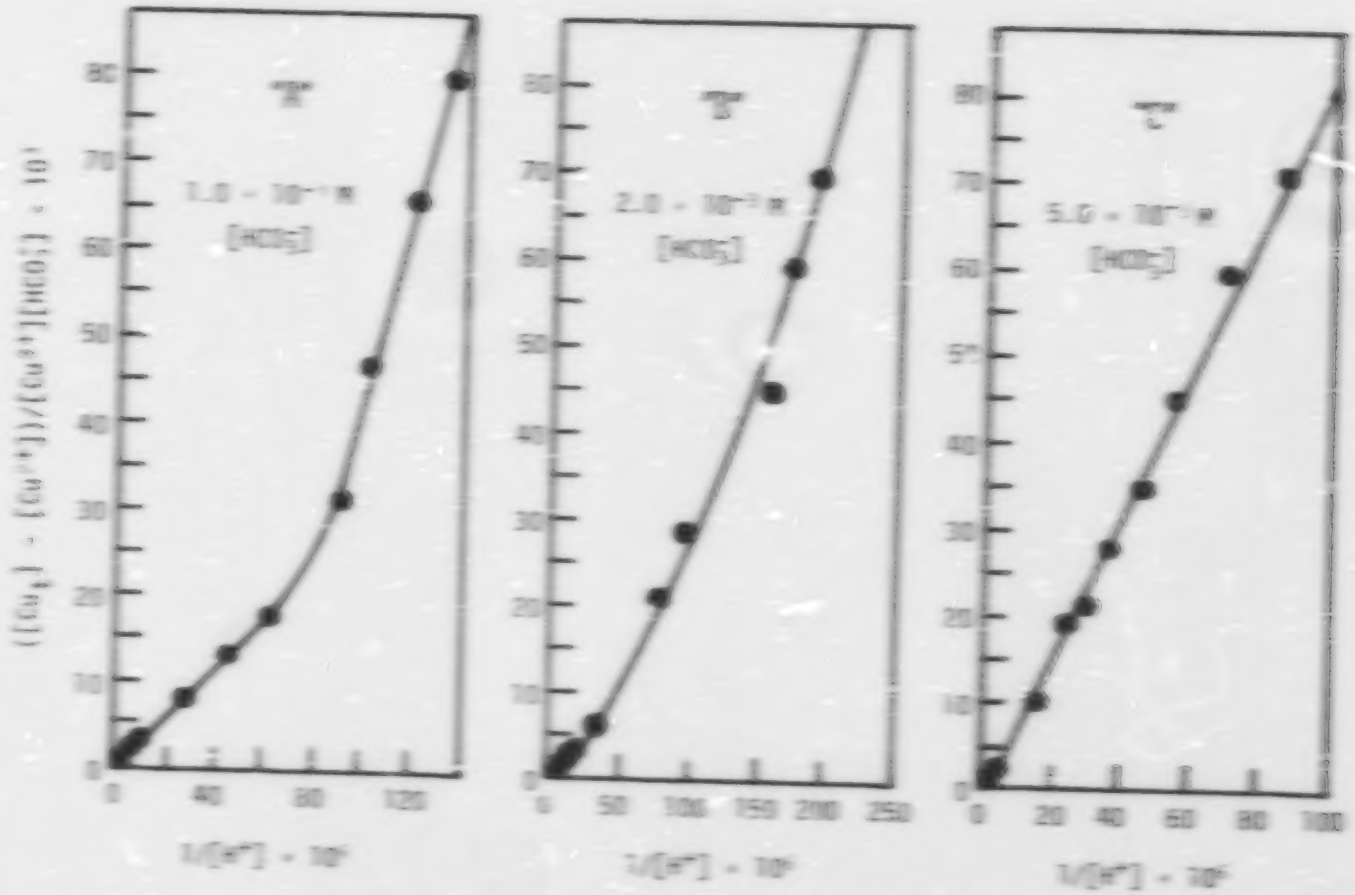


Figure 10. Graphical representation of Cu^{++} - carbonate ion titrations.

The value of (γ_{CuCO_3}) is 1.0 since $CuCO_3$ is an uncharged species, and the other activity coefficients were taken from Kielland[83] and Stiff [82].

The stability constant of the complex species $CuCO_3$ can be evaluated from the following equations:

$$Cu^{2+} + HCO_3^- \rightleftharpoons CuCO_3 + H^+ \qquad (49)$$

and $$CO_3^{2-} + H^+ \rightleftharpoons HCO_3^-, \quad K_3' = [a_{HCO_3^-}]/[a_{CO_3^{2-}}][a_{H^+}] \qquad (50)$$

where ^{a} denotes activity. In summing equations (49) and (50),

$$Cu^{2+} + CO_3^{2-} \rightleftharpoons CuCO_3 \qquad (51)$$

$$K_4' = \frac{[a_{CuCO_3}]}{[a_{Cu^{2+}}][a_{CO_3^{2-}}]} = K_2'K_3'$$

or $$pK_4' = pK_2' + pK_3', \qquad (52)$$

The equilibrium and stability constants for this reaction at different bicarbonate ion concentrations are given in Table 4.

By using a value of -10.3 for pK_3' as reported in the literature[84], the pK_4 values determined from our experimental data were found to be -7.3. We realise that pK_4' determined from our results is higher than those reported by Stiff[82] and by Silman[81]. Nevertheless, our results were quite reproducible as indicated in Table 4.

Table 4: EQUILIBRIUM AND STABILITY CONSTANTS

Bicarbonate concentration [M]	pK_2'	pK_4'
1.0×10^{-3}	2.94	-7.3
2.0×10^{-3}	2.96	-7.3
5.0×10^{-3}	3.00	-7.3

SECTION VI

DEVELOPMENT OF THE BIOASSAY SYSTEM

A vast amount of literature on metal toxicity is based on bioassay experiments. Many of the available data resulted from studies with limited objectives, e.g., the determination of the effect of waste discharge on a receiving water. The remaining data are for the most part ambiguous and difficult to interpret. In fact, most bioassay data, if not all, have resulted from studies based on limited or no consideration of the underlying chemistry. Regrettably, these data are the only information available on which water quality criteria can be based. At present, there is a pressing need for a better data base -- one which is founded upon scientifically conceived and rigorously defined bioassay studies.

The chief task in this study was the design of a bioassay system in which physicochemical and biological interrelationships were accounted for and in which independent variables could be rigorously controlled. The main interrelationships that need to be considered in the study of metal toxicity are illustrated in Figure 11 in terms of (1) physicochemical characteristics of bioassay water, (2) concentration of metal species, (3) metal uptake by test organism, and (4) biological response. In this schematic representation the central factor is the physicochemical characteristics of the bioassay water. These properties will dictate the concentration of the toxic metal species in the bioassay water (arrow 1-2) and influence metal uptake by the test organism (arrow 1-3) and the biological response (arrow 1-4). The concentration of toxic

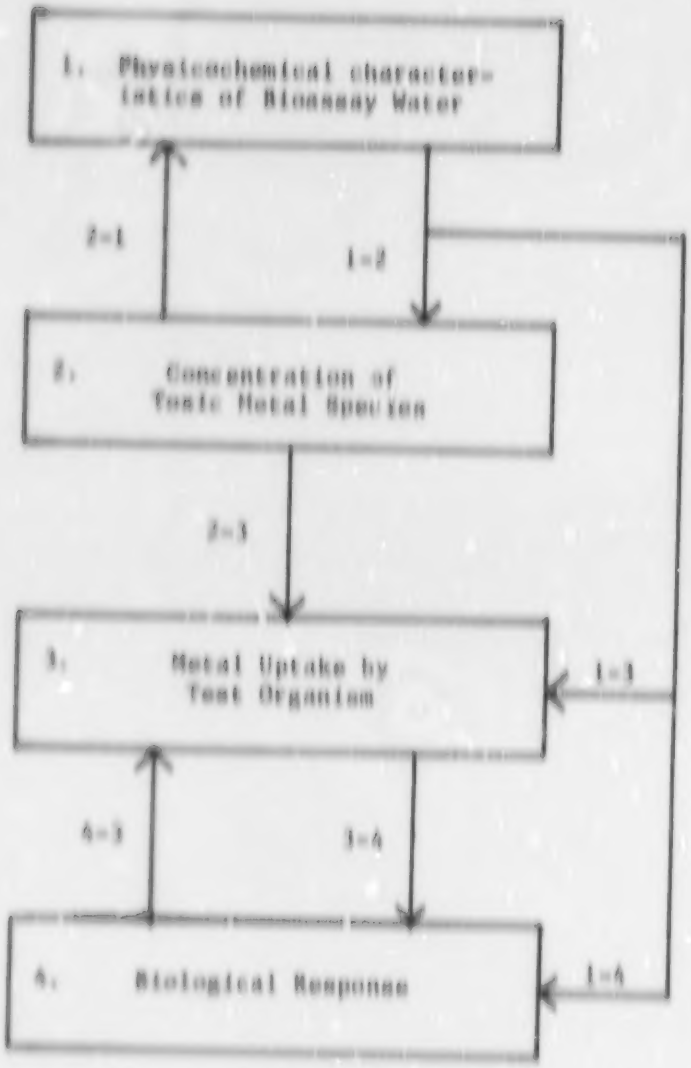


Figure 11. Physicochemical and biological interrelationships in bioassay experiments.

metal species will in turn influence the physicochemical characteristics of the bioassay water (arrow 2-1) through adsorption, complexation, ion exchange, and other reactions of the metal species. Metal uptake by the test organism is largely dependent on the concentration of the different species of the toxic metal (arrow 2-3). The biological response of the test organism is determined by the rate and magnitude of the metal uptake (arrow 3-4). In turn the physiological status of the test organism as indicated by the biological response will significantly influence metal uptake (arrow 4-3).

Bioassays have been conducted in one of two ways: (1) in static systems, usually to establish the minimum dilution of an industrial waste in which fish or other aquatic organisms survive, or (2) in dynamic systems, either in the laboratory or in the field, to determine the toxicity of a given material. In neither approach, especially where metals are concerned, has the investigator been able to effectively control the chemical and physical variables to permit his results to be applied to other situations. It is virtually impossible to control all the variables in a laboratory bioassay. Nevertheless, field experiments are not the complete answer. Although the field test indicates the toxicity of the metal under consideration in the environment, the investigator cannot use this method to understand the role of each of the important physicochemical variables in producing the observed effect. The main physicochemical variables that need to be controlled are hydrodynamics, temperature, ionic strength, light, dissolved oxygen, pH, alkalinity, and the chemical composition of the bioassay medium. Some of these variables, such as temperature and light, are usually considered by most investigators, but others are rarely taken into account.

This section does not review the physical and chemical variables affecting toxicity. A critical review of some of these factors has been published elsewhere[85].

CHEMICAL SPECIES OF METAL TOXICANTS

Few bioassays have been conducted so that the results can be interpreted in terms of the toxicity of specific forms of the metal. No studies have been conducted that controlled pH, alkalinity, and hardness independently. Mount[30] investigated the effect of pH and total hardness on the acute toxicity of zinc to fish. Hardness was controlled at values of 50, 100, and 200 mg/l $CaCO_3$ by mixing limestone spring water and deionized water. The pH was controlled by the addition of strong acid or base. Addition of the strong acid or base will, besides controlling the pH, change the alkalinity of the system[26]. For example, at a hardness of 100 mg/l the alkalinities were 16, 56, and 102 mg/l $CaCO_3$ at pH 6, 7, and 8, respectively. The change in the alkalinity of the system is due to the rapid establishment of chemical equilibrium between the solution and the atmosphere.

Most investigators consider alkalinity and hardness to be related since, in nature, waters of high alkalinity tend also to be high in hardness. These two properties are, however, basically not related since alkalinity refers to the system's acid-neutralising capacity and hardness refers to its divalent metal concentration or its capacity to complex with EDTA. A solution of calcium chloride will have a high level of hardness and a low level of alkalinity, whereas a sodium carbonate solution will have a low hardness and a high alkalinity.

The concentrations of carbonate, bicarbonate, and carbon dioxide in an aquatic system are of importance primarily because of their roles in pH control, complexation of metals, and control of metal solubility. Below is presented a simple method, developed by Roberts and Allen[86], for the control of total carbonate or total alkalinity and pH in the bioassay experiment. This technique is applicable to natural as well as synthetic waters.

The formation of significant quantities of metal carbonate or hydroxy complexes will affect the chemical relationships presented. The amount of base and the partial pressure of carbon dioxide required to establish

a desired set of conditions can be predicted if the appropriate equilibrium constants in the equations presented are included.

EQUILIBRIUM RELATIONSHIPS IN THE CARBONIC ACID SYSTEM

Equilibrium Relationships of Total Alkalinity and pH

The total alkalinity of a sample is defined as its acid-neutralizing capacity[36]. This can be expressed as:

$$\text{Total Alkalinity} = 2[CO_3^{2-}] + [HCO_3^-] + [OH^-] - [H^+] \qquad (53)$$

where the brackets indicate concentrations, and the alkalinity is expressed in equivalents per liter. For a sample exposed to the atmosphere the concentrations of carbonate and bicarbonate ions are related to the partial pressure of carbon dioxide (P_{CO_2}) by the following relationships:

$$[CO_3^{2-}] = \frac{10^{-18.15} P_{CO_2}}{[H^+]^2} \qquad (54)$$

and

$$[HCO_3^-] = \frac{10^{-7.93} P_{CO_2}}{[H^+]} \qquad (55)$$

By substituting these relationships in equation (54) the following equation results:

$$[\text{Total Alkalinity}] = \frac{10^{-17.85} P_{CO_2}}{[H^+]^2} + \frac{10^{-7.93} P_{CO_2}}{[H^+]} + \frac{10^{-14.00}}{[H^+]} - [H^+] \qquad (56)$$

In this expression for total alkalinity the pH and partial pressure are the variables, but this expression will always equal zero unless a base is added to the solution. This can best be shown by an example.

In a solution of sodium bicarbonate, sodium carbonate, or sodium hydroxide, the following anion-cation balance will hold:

$$[Na^+] + [H^+] = 2[CO_3^{2-}] + [HCO_3^-] + [OH^-] \qquad (57)$$

Rearrangement gives:

$$[\text{Total Alkalinity}] = [Na^+] = 2[CO_3^{2-}] + [HCO_3^-] + [OH^-] - [H^+] \qquad (58)$$

The total alkalinity is equal to the amount of base in solution and will equal zero if no base is present.

We can now substitute the sodium ion concentration for total alkalinity in equation (57), which on rearrangement yields an expression for the partial pressure of carbon dioxide as a function of the total alkalinity (or sodium ion concentration) and the pH:

$$P_{CO_2} = \frac{([Na^+][H^+] + [H^+]^2 - 10^{-14.00})[H^+]}{10^{-17.05} + 10^{-7.82}[H^+]} \qquad (59)$$

This equation is important because it shows that the $[H^+]$ can be controlled by the partial pressure of carbon dioxide and the amount of base added to the solution. Both of these variables can be controlled by the investigator.

A computer program for the computation of the P_{CO_2} required to establish equilibrium at any pH and total alkalinity[86] is presented in Appendix B. Results for 10^{-1}, 10^{-2}, and 10^{-3} eq/l for the pH range 6.0 to 12.0 are presented in Appendix B. The results computed and the titles given them in the computer output are total carbonate concen-

tration (TCO3), the logarithm of the total carbonate concentration (LOG TCO3), the logarithm of the partial pressure of carbon dioxide (LOG PCO2), and the logarithms of the molar concentrations of CO_3^{2-}, HCO_3^-, and H_2CO_3 (LOG CO3, LOG HCO3, and LOG H2CO3, respectively).

Equilibrium Relationships of Total Carbonate and pH -- The total carbonate, $[CO_3^{2-}] + [HCO_3^-] + [H_2CO_3]$, is also controlled by the partial pressure of carbon dioxide and the pH according to the following relationship:

$$[\text{Total Carbonate}] = \frac{10^{-10.15}p_{CO_2}}{[H^+]^2} + \frac{10^{-7.82}p_{CO_2}}{[H^+]} + 10^{-1.47}p_{CO_2} \quad (60)$$

where

$$[H_2CO_3] = 10^{-1.47}p_{CO_2} \quad (61)$$

Solving equation (60) for the partial pressure of carbon dioxide,

$$p_{CO_2} = \frac{[\text{Total Carbonate}][H^+]^2}{10^{-10.15}+10^{-7.82}[H^+]+10^{-1.47}[H^+]^2}, \quad (62)$$

an expression is obtained that can be used to calculate the necessary partial pressure of carbon dioxide for any desired pH and total carbonate concentration. The concentration of sodium ion necessary to establish the desired conditions is given by equation (60), which can be rewritten

$$[Na^+] = \frac{(10^{-17.85}+10^{-7.82}[H^+])p_{CO_2}}{[H^+]^2} + \frac{[H^+]^2-10^{-14.00}}{[H^+]} \quad (63)$$

Addition of $NaHCO_3$, Na_2CO_3, or NaOH is necessary so that the total carbonate and pH may be independently varied.

Calculation Procedures for Synthetic Waters --

Case I: Total Alkalinity and pH Control.

The procedure for controlling total alkalinity and pH in solutions made from distilled or deionized water is very simple:

1) Decide what total alkalinity is desired and add that molar concentration of $NaHCO_3$, NaOH, or half as much Na_2CO_3 to the solution.
2) Mix carbon dioxide with air or a nitrogen-oxygen mixture and bubble the final mixture through the solution, varying the amount of carbon dioxide until the desired pH is obtained and maintained in equilibrium.

The pH may be varied simply by adjustment of the amount of carbon dioxide in the mixture, and the alkalinity will remain constant at any pH.

Case II: Total Carbonate and pH Control.

The control of total carbonate and pH involves the following calculation:

1) Calculate the amount of $NaHCO_3$, NaOH, or Na_2CO_3 necessary for the desired total carbonate and pH from the following equation which can be derived from equations 62 and 63.

$$[Na^+] = \frac{[\text{Total Carbonate}](10^{-17.05}+10^{-7.82}[H^+])}{10^{-10.15}+10^{-7.82}[H^+]+10^{-1.47}[H^+]^2} - \frac{[H^+]^2-10^{-14.00}}{[H^+]} \qquad (64)$$

2) After the addition of the sodium ion, the pH is adjusted with a mixture of carbon dioxide and air or a nitrogen-oxygen mixture as described in the total alkalinity case. Once the desired pH has been obtained, the total carbonate concentration should also be at the desired level. These calculations and additions of base must be done for each pH and total carbonate concentration.

Figures 12 and 13 show the partial pressures of carbon dioxide and the amounts of base necessary to maintain total alkalinities (eq/l) or total carbonate concentrations (moles/l) of 10^{-2} and 10^{-3}. There is a minimum pH that can be maintained for any designated total carbonate concentration and a maximum pH that can be obtained for any desired alkalinity. The maximum pH decreases by one unit as the alkalinity decreases tenfold. In Figures 12 and 13 the maximum pH decreases from 12 to 11 as the total alkalinity decreases from 10^{-2} to 10^{-3} eq/l. The minimum pH that can be maintained in the case of total carbonate control is not directly proportional to the total carbonate concentration. As the total carbonate decreases from 10^{-2} to 10^{-3} M the minimum pH increases from 6.0 to 6.7.

Limitations -- Although it is theoretically possible to achieve any pH and total alkalinity or total carbonate by this procedure, practical constraints limit its usable range. At low pH's and high total alkalinity or total carbonate, high concentrations of free carbon dioxide may be lethal to fish. At high pH's the high ionic strength present in the control of total carbonate may prove deleterious; also it may be difficult in practice to control the low carbon dioxide pressure required to maintain a high pH. These problems should not develop in the control of bioassay chemical conditions because extreme values of pH and total alkalinity or total carbonate would not be desired.

Generally, the predominant factor in controlling pH and alkalinity is the carbonate system. However, certain bioassay conditions may significantly alter the total alkalinity, total carbonate, and pH in vivo. For example, high hardness, alkalinity, and pH may cause carbonate precipitation and reduce the total alkalinity. High concentrations of metal toxicants such as lead may also result in the formation of metal hydroxides, which would lower the alkalinity. These conditions can be recognized if the pH obtained is significantly lower than the predicted value.

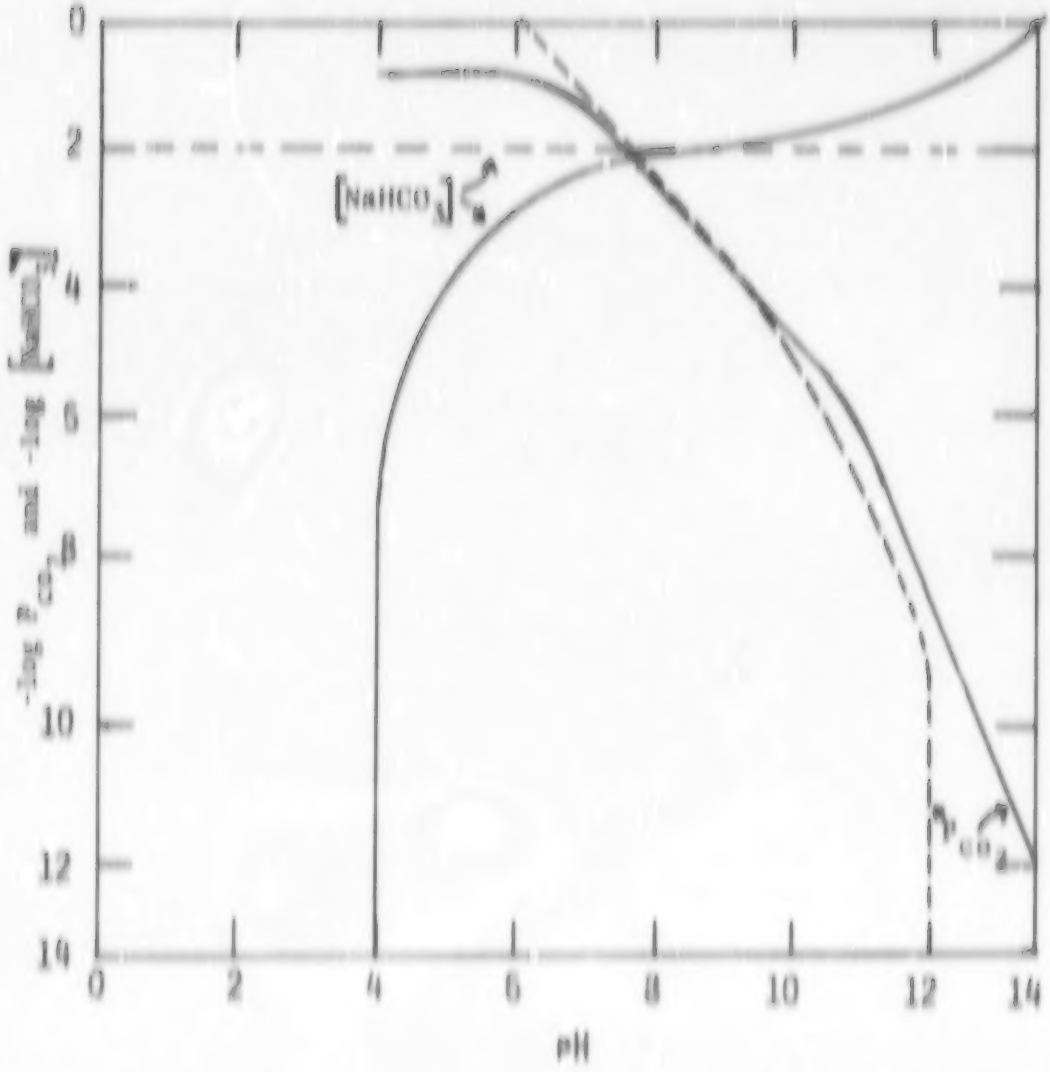


Figure 12. Partial pressure of carbon dioxide and amount of sodium bicarbonate (moles/liter) required to maintain total carbonate = 10^{-2} molar (solid line) or total alkalinity = 10^{-2} equivalents/liter (dashed line). Total carbonate cannot be below pH 4.0, and total alkalinity cannot be above pH 12.0.

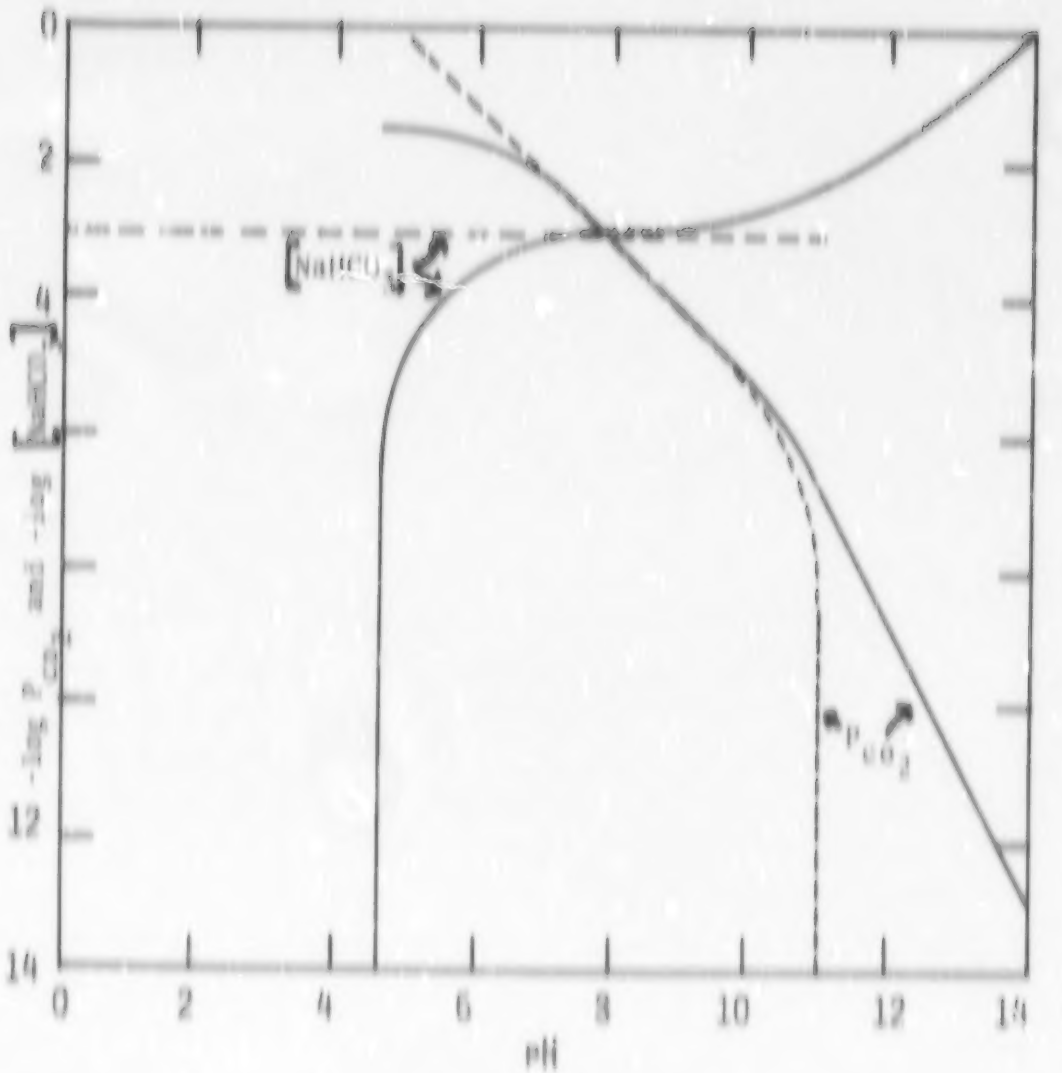


Figure 13. Partial pressure of carbon dioxide and moles/liter sodium bicarbonate required to maintain total carbonate = 10^{-2} molar (solid line) or total alkalinity = 10^{-2} equivalents/liter (dashed line). Total carbonate cannot be controlled below pH 4.7 and total alkalinity cannot be controlled above pH 11.0.

SECTION VII

DESIGN OF BIOASSAY EXPERIMENT

As stated earlier, it is essential that the chemical factors affecting copper toxicity to fishes be controlled. Although numerous reports deal with the toxicity of copper, none includes an account of copper species, e.g., free copper ions, inorganic and organic copper complexes, and colloidal dispersions of copper species.

In our bioassay test facility the following variables were controlled: (1) ionic strength; (2) inorganic ligands: hydroxide and carbonate; (3) calcium; and (4) copper species. The following variables were measured during the testing of the bioassay facilities: (1) toxicity as measured by standard bioassay analysis techniques; (2) concentration of copper species including free, labile, nonlabile, particulate, and total; and (3) characteristics of the bioassay water including pH, calcium, carbonate, dissolved oxygen, and turbidity. The system design insures that the bioassay water is of the desired chemical composition and that it does not change during the course of the bioassay. This stable condition is accomplished by using a synthetic bioassay water that is allowed to reach chemical equilibrium before the beginning of the bioassay, by using continuous flow bioassays, and by measuring the chemical composition of the bioassay water throughout the test. Special precautions must be taken to insure that water samples do not change between collection and analysis.

Since the attainment of chemical equilibrium is often a slow process, the bioassay water must be prepared and allowed to reach equilibrium before exposing the test organism to it. An example of the slow at-

tainment of equilibrium is illustrated in Figure 14. Lead, copper, and zinc nitrates were added at an initial concentration of 2.5×10^{-6} M in an aqueous solution of 10^{-3} eq/l total alkalinity and pH 9. Pulse polarographic measurement of copper, lead, and zinc as a function of time indicates a slow process of metal transformation[87]. Considerable time is needed to assure the attainment of equilibrium. Consequently, the common practice of adding the toxic agent immediately before exposure of the test organism should be viewed with skepticism. Needless to say, analyses conducted to verify the existence of a given metal species at steady-state conditions may not be valid since measurements are invariably made much later than the moment of exposure. This discrepancy limits our ability to extrapolate from data obtained with "diluters," such as those described by Mount and Warner[88] and Esvelt and Connors[89].

Flow bioassays were deemed necessary to insure that chemical conditions did not change during the bioassay. In addition to the usually cited deficiencies of static bioassays (loss of toxicant through biological uptake or adsorption and the build-up of toxic metabolic waste products), waste products released by the test organism may react with copper resulting in changing the chemical species of the toxicant present.

HATCHERY AND BIOASSAY SYSTEM

Fish Hatchery

A fish hatchery was established in which the necessary test organisms (juvenile guppies, Lebistes reticulatus) were raised and conditioned to the required environmental conditions before their use in a bioassay. Over 600 adult guppies were maintained in two 70-gal. aquaria. Aquatic plants covered the surface to provide cover for the young. Illumination was maintained 18 hours a day, and newborn fish were removed daily and transferred to 5-gal. aquaria.

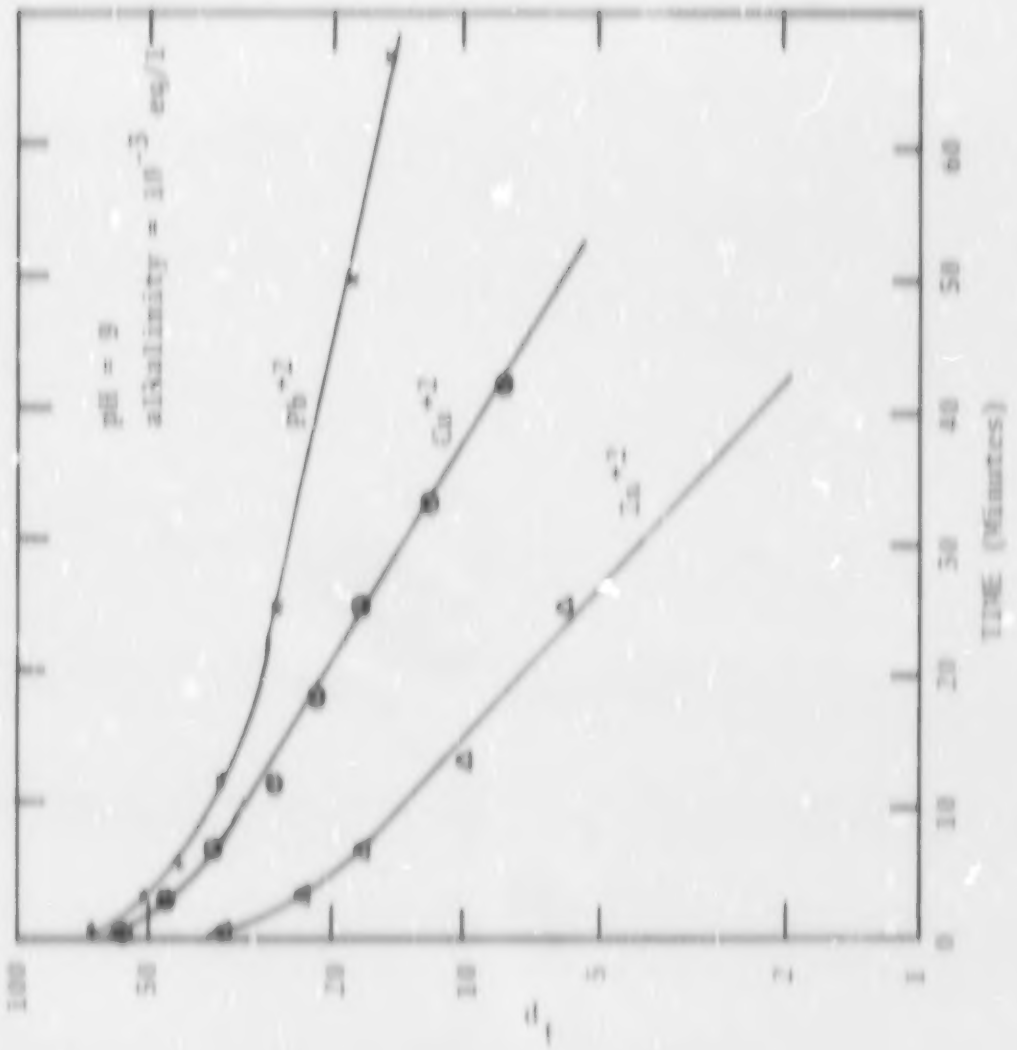


Figure 14. Change of ASV signal for Zn^{+2}, Cu^{+2}, and Pb^{+2} with time.

The juvenile fish to be used in the bioassay were approximately 10 days to 2 weeks old. Guppies in this age group are no more susceptible to copper poisoning and stress than older fish. This factor was demonstrated during static bioassays in which two groups of fish of different ages were subjected to identical copper concentrations. In all tests the older fish died at the same rate as the younger fish. Because it is easier to maintain a large number of younger fish, the 10-day to 2-week-old guppies were used as experimental organisms.

Ann Arbor tap water was used for the hatchery water supply. It was dechlorinated by passage through a charcoal filter, and the pH was maintained in the 7.1 - 7.4 range. The conditioned water was held in a 250-gal. aluminum tank; the water in the 70-gal. aquaria was continuously exchanged. The water supply system and hatchery are shown schematically in Figure 15. Water level in the 250-gal. reservoir was maintained by a float valve. The pH was measured, and the output from the pH meter was used as the input for a controller with a built-in recorder, enabling the record to be checked for pH deviations outside the acceptable range. The controller was used to activate two peristaltic pumps to dispense acid and base. Although this system is capable of maintaining a narrower pH span, the limits were set to provide a pH within a tolerable range without requiring the pumps to be activated excessively. At times only a single pump delivering acid was utilized; the high pH (greater than pH 10) of the Ann Arbor water, as compared to the desired pH, eliminated the need for addition of base.

Fish Conditioning

The fish-conditioning system, shown in Figure 16, included a 250-gal. equilibrium tank that contained water of the required chemical composition. A heater and thermoregulator on a styrofoam float guided by plastic rods maintained the conditioning water at 25° C. Water from the equilibrium tank flowed through a series of 5-gal. aquaria containing the fish to be conditioned for a bioassay. The aquaria were equipped with heaters and constant-level siphons.

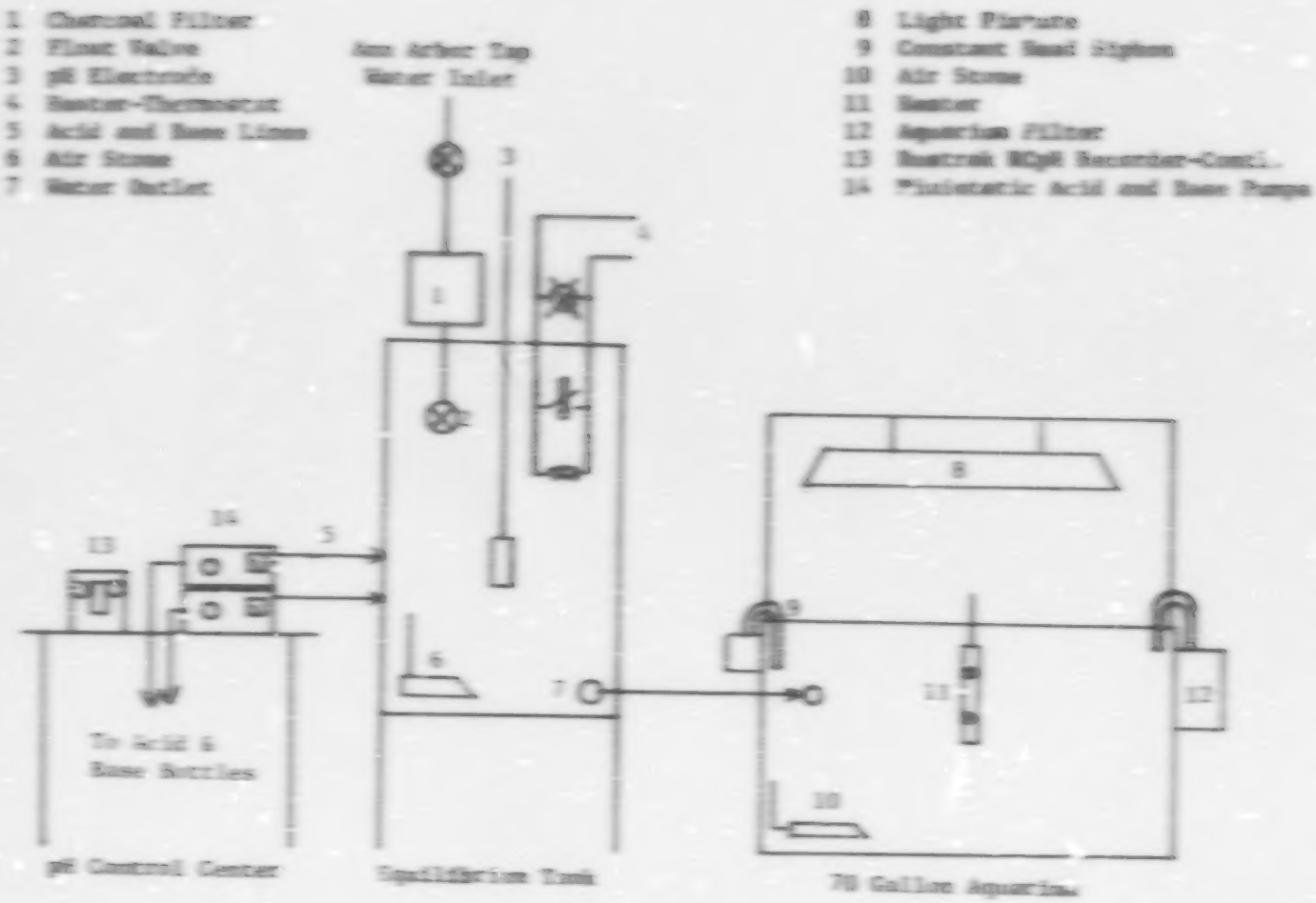


Figure 15. Schematic diagram of hatchery.

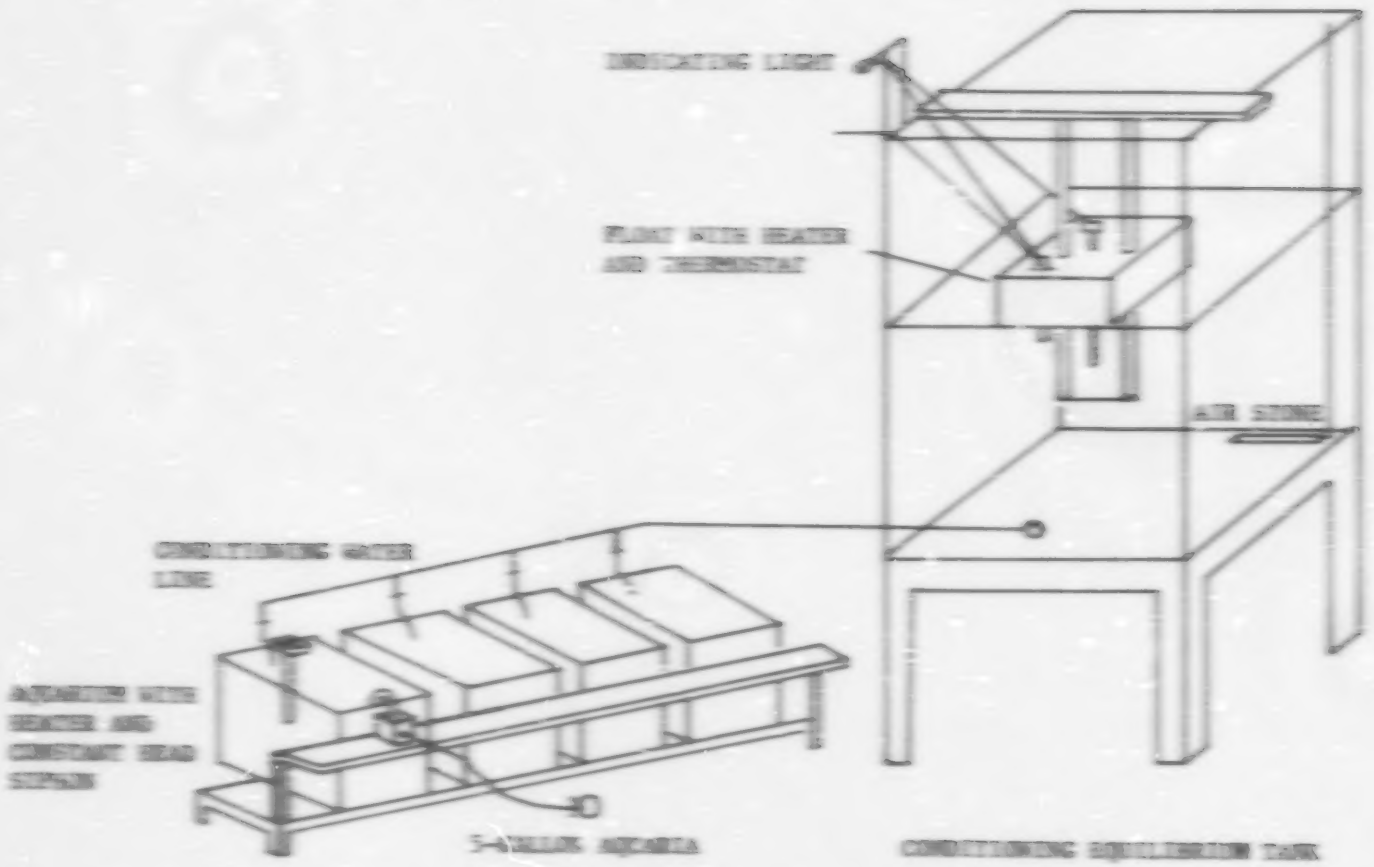


Figure 16. Schematic diagram of fish-conditioning area.

The fish were acclimated for 10 days. Two days before the beginning of a bioassay, the conditioned fish were transferred to their appropriate bioassay chambers. The conditioning water was then diverted to these chambers. This procedure eliminated stress and death of fish due to damage or weakening by transfer from the conditioning aquaria to the bioassay chambers.

Bioassay Water

The test solutions were prepared several days in advance of their use to permit the solutions to reach chemical equilibrium and to allow time for chemical analyses of the water before beginning a bioassay. The required chemicals were added to charcoal-filtered, deionized water contained in 250-gal, fiberglass-covered plywood tanks. Sodium bicarbonate was added to the water to control the pH. Then, carbon dioxide and air were mixed in a 3 x 1 x 1-foot baffled fiberglass-covered plywood mixing box. The gas mixture was bubbled through the test water; large aquarium stones were used for gas dispersion. The partial pressure of carbon dioxide was adjusted until the desired pH of the water was obtained. The theoretical considerations of the control of pH in this manner have been discussed previously.

To control hardness calcium sulfate was added to the equilibrium tanks. Magnesium sulfate, sodium chloride, and potassium sulfate, all necessary ions for proper physiological function of the fish, were also added to the water. Sodium sulfate was added to obtain constant ionic strength for the experiments. The concentration of each of the chemicals was: $[Ca^{2+}] = 2 \times 10^{-4}$ M; $[CO_3^{2-}] = 1 \times 10^{-3}$ M; $[Cl^-] = 30$ ppm; $[K^+] = 4$ ppm; and $[Mg^{2+}] = 4$ ppm.

Randomization and Exposure of Fish for Bioassays

In the bioassay, the fish must be assigned to the bioassay chambers in a random manner since the fish removed from the aquarium may be the weakest. Randomization prevents placing all the weakest fish in a single chamber. A computer program was written that gives a random

sequence for assigning fish to bioassay chambers. Fish were placed in 1-quart plastic containers until each container had one fish, then a second fish was added to each chamber. This process was continued until the required number of fish had been added to each container. The fish in each container were then transferred to the identically designated bioassay chamber. Plastic containers were used because they could be clustered in a small area around the aquarium. This arrangement minimized the time required to accomplish the randomization and also minimized the time that a fish had to be kept in the net.

The computer program (Table 5) provides the random sequence for adding the fish to the chambers. The variables L and M in the program are, respectively, the number of bioassay chambers to be used in the experiment and the total number of fish to be placed in each chamber. The computer output for 12 bioassay chambers and 20 fish per chamber has been included (Table 6). This computer program has as its essential part the subroutine RANDU which generates the random numbers. This subroutine produces the same results as the subroutine of the same name available in IBM's Scientific Subroutine Package/System 360. The subroutine has been rewritten for use on the IBM 1130 computer and was supplied by Professor L.S. Whitlock, Department of Biostatistics, School of Public Health, The University of Michigan.

After all the fish were randomized to their respective chambers, they were allowed to acclimatize to a 30 ml/min flow of conditioning water for approximately 48 hours. Then the actual test-water solutions containing copper flowed into the chambers at about 30 ml/min. The bioassay continued for 96 hours. At 8-hour intervals the chambers were checked to measure various characteristics including pH, temperature, resistivity, flow rate, and dissolved oxygen. Also, at these times dead fish were removed from the chambers. For experimental purposes lack of pectoral fin movement indicated death. The time and the day of each fish death were recorded on data sheets throughout the 96-hour experiment.

Table 5.

RANDOM SEQUENCE FOR ADDING FISH TO THE CHAMBERS

```
      DIMENSION J(14)
      WRITE(1,200)
200   FORMAT(' GIVE ME 2 ODD RANDOM NUMBERS IN (I4,1X,I4)FORMAT,'/
     1' BOTH NUMBERS SHOULD BE LARGER THAN 4000')
      READ(6,300)IX1,IX2
300   FORMAT(I4,1X,I4)
      IX1=IX1/2*2+1
      IX2=IX2/2*2+1
      WRITE(5,400)
400   FORMAT(1H1,9X,'RANDOM ORDER FOR PLACING FISH IN BIOASSAY CHAMBERS'
     1///' FISH NUMBER    1A  1B  2A  2B  3A  3B  4A  4B  5A  5B  6A  6B
     2 7A  7B'/1X,68('*'))
      L=12
      M=20
      DO 60 K=1,M
      DO 5 I=1,L
5     J(I)=-99
      NC=0
40    CALL RANDU(IX1,IX2,R)
      DO 20 I=1,L
      IF(R*L-I)10,20,20
10    IF(J(I))30,40,40
30    J(I)=NC+1
      NC=NC+1
      IF (NC-L)40,60,60
20    CONTINUE
60    WRITE(5,100)K,(J(I),I=1,L)
100   FORMAT(1H0,6X,I3,3X,1414)
      CALL EXIT
      END
```

Table 6.

RANDOM ORDER FOR PLACING FISH IN BIOASSAY CHAMBERS

FISH NUMBER	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B	6A	6B	7A	7B
1	4	8	9	10	3	12	5	3	7	11	1	6		
2	12	8	4	2	1	6	11	7	5	9	10	3		
3	2	5	4	12	1	8	10	3	6	11	9	7		
4	2	9	11	3	5	7	12	10	6	4	1	8		
5	2	1	9	3	5	10	11	6	8	4	12	7		
6	10	3	8	7	6	11	5	9	12	1	2	4		
7	6	1	8	10	5	7	4	2	3	12	9	11		
8	12	3	7	1	4	6	9	11	8	2	5	10		
9	11	12	1	8	3	2	4	5	10	9	6	7		
10	11	1	5	8	4	7	3	5	12	9	10	2		
11	11	2	3	10	5	8	9	1	6	12	7	4		
12	3	12	5	1	4	9	8	2	10	6	11	7		
13	11	10	6	8	7	3	9	1	5	4	2	12		
14	8	1	12	3	7	10	6	4	5	11	3	9		
15	9	1	12	7	6	8	11	5	3	10	4	2		
16	5	11	3	2	4	12	8	10	9	6	7	1		
17	6	1	3	4	5	10	2	8	12	9	11	7		
18	8	2	4	12	5	1	6	7	9	3	11	10		
19	3	2	12	1	11	4	5	7	9	8	10	6		
20	2	3	7	1	9	8	12	10	4	5	6	11		

Exposure Facility

The experimental facility is shown in Figures 17 and 18. The test water flowed from the equilibrium tank to the bioassay chambers through 25-foot coils of stainless steel tubing contained in three large water baths connected by siphons and a pump. Medical-grade silicone tubing was used for connections. The bioassay chambers were contained in the water bath to permit better temperature control and in case the disposal of effluent water. Excess water in the bath was removed by a standpipe. The temperature of both the bath and the water contained in the exposure chambers could be regulated with a half-degree precision.

The exposure chambers (Figure 19) were fabricated from glass and silicone sealant. These chambers had a volume of approximately 1 liter, and the flow of water through the chambers was baffled by the placement of a plastic screen across each end. The flow pattern through the chamber, as determined by dye tracing, was even.

TOXICITY EXPERIMENTS

Three bioassay experiments were conducted for the sole purpose of assessing the validity of the newly developed concepts and techniques of chemical control. The time required to conduct one bioassay was between 2 and 3 weeks. Physicochemical data for pH, temperature, electrical resistance, hardness, alkalinity, and total carbonate are presented in Table 7. Alkalinity was determined by potentiometric titration, and total carbon was determined by total carbon analysis.

Data for bioassays at pH 8.10, 7.00, and 5.67 (bioassays A, B, and C, respectively) are presented in Table 8. Percentage survival was determined at 48 hours; in most cases the mortality at 96 hours' exposure was so high that the data were not usable. In bioassay A all fish died at the highest copper concentration. In addition, percentage survival was lower at 130 ppb total copper than at 378 ppb. In bioassays B and C there were no anomalies in the data.

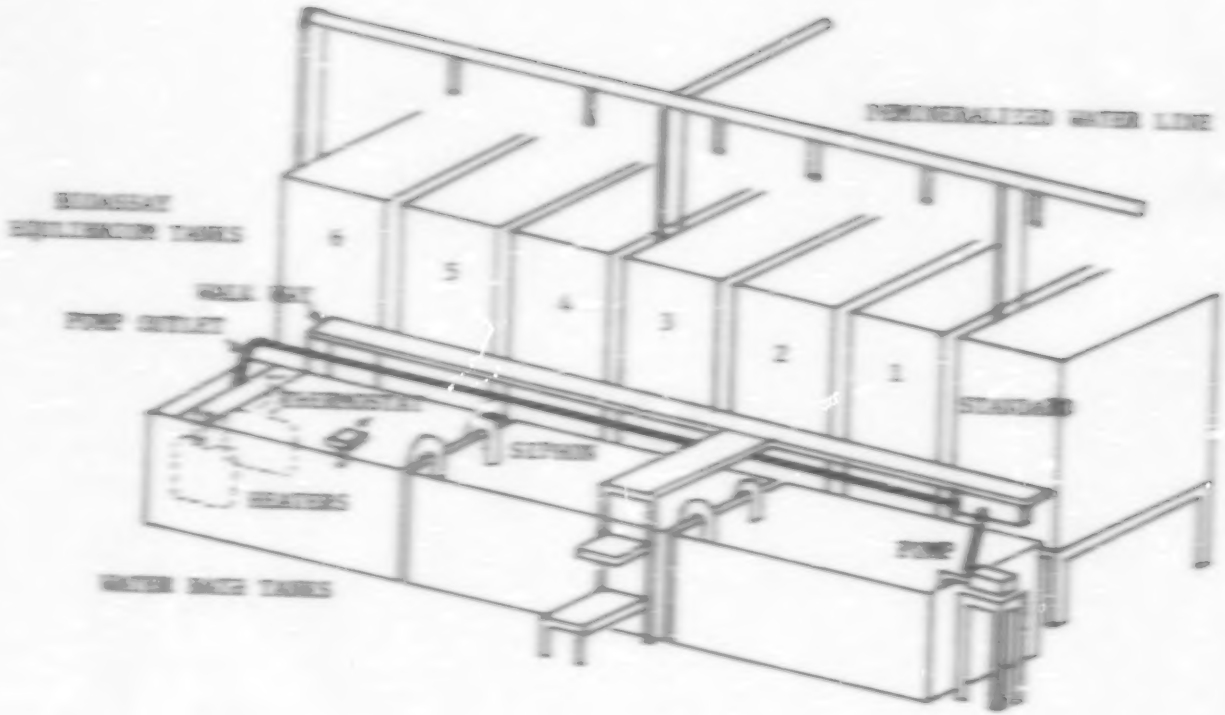


Figure 17. Schematic diagram of bioassay area.

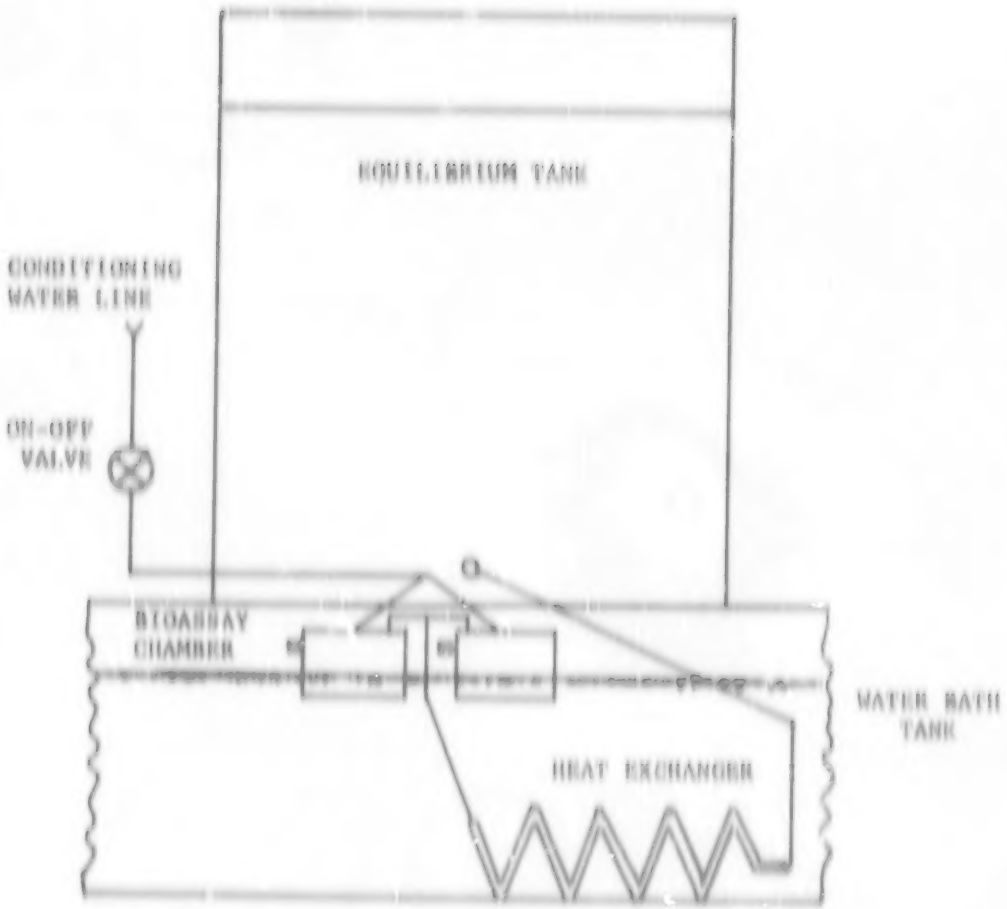


Figure 14. Schematic diagram of bioassay system.

MATERIALS: DOUBLE STRENGTH GLASS
PLASTIC SCREENING
BONDED WITH SILICONE RUBBER SEALER

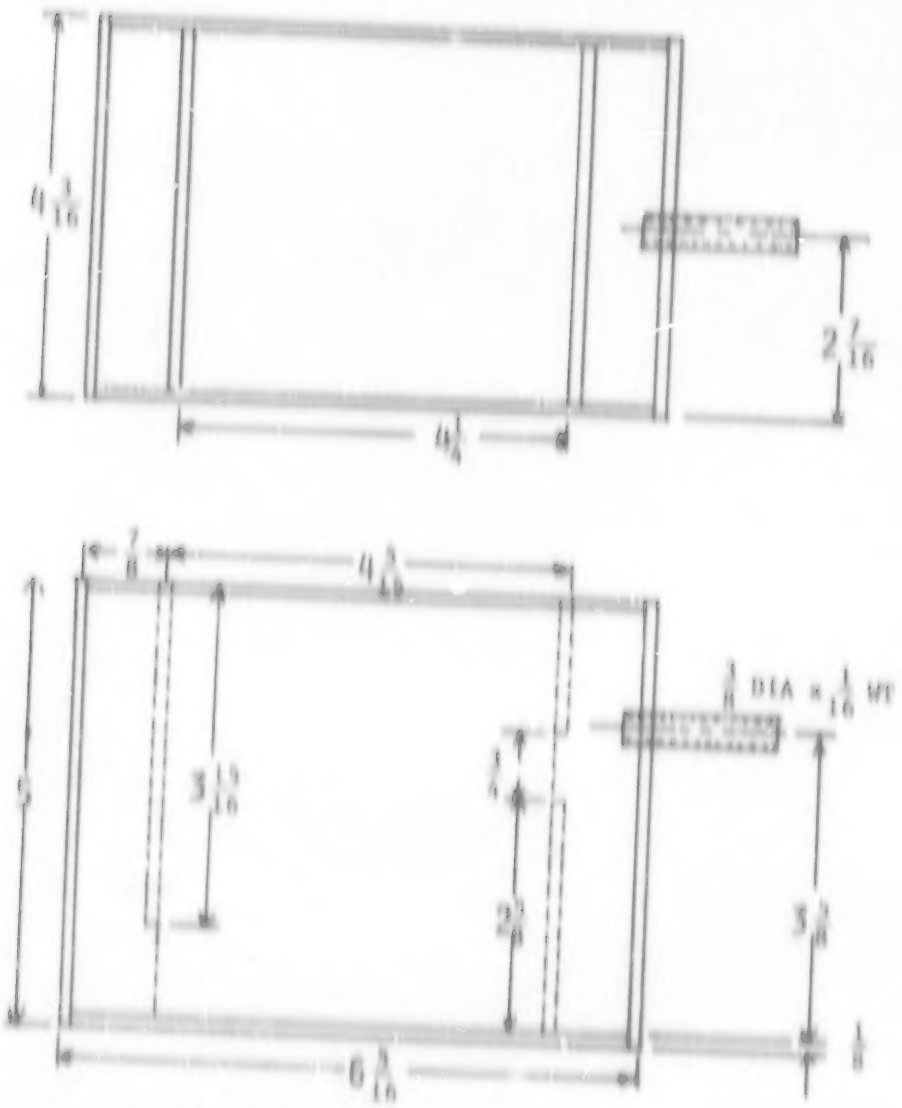


Figure 19. Bioassay exposure chamber.

Table 7. SUMMARY OF BIOASSAY HISTOCHEMICAL DATA

Variable	Bioassay A			Bioassay B			Bioassay C		
	$\bar{X}^a$	n	s	$\bar{X}^a$	n	s	$\bar{X}^a$	n	s
pH	8.10	67	0.12	7.00	66	0.06	5.67	120	0.33
Temperature (°C)	24.8	56	0.2	24.7	19	0.3	24.9	38	0.2
Electrical Resistance (ohms)	3150	46	78	3200	19	51	4007	84	129
Hardness (meq/l)	0.404	6	0.010	0.384	6	0.092	0.412	6	0.012
Alkalinity (meq/l)	0.975	6	0.022	0.769	6	0.023	0.130	6	0.002
Total Carbonate: (mM) Measured	1.95	6	0.035	0.87	6	0.026	0.95	6	0.036
(mM) Calculated from Alkalinity	0.98	—	—	0.97	—	—	0.75	—	—

[a] Arithmetic average

TABLE 6. SUMMARY OF FISH SURVIVAL DATA AND COPPER MEASUREMENTS[a]

	Statistic	Total Copper	Soluble Copper	Particulate Copper	Free & Labile Copper	Percentage Survival
Bioassay A (pH 8.10)	$\bar{X}$	<1	---	---	---	100.0
	N	1	---	---	---	
	σ	---	---	---	---	
	$\bar{X}$	42	26	12	6	92.5
	N	4	2	2	2	
	σ	3	8	9	2	
	$\bar{X}$	70	32	42	10	42.5
	N	4	2	2	2	
	σ	6	2	5	1	
	$\bar{X}$	130	56	77	22	7.5
	N	4	2	2	2	
	σ	11	4	4	1	
	$\bar{X}$	278	78	131	39	15.5
	N	4	2	2	2	
	σ	46	23	16	1	
	$\bar{X}$	343	154	229	91	0.0
	N	4	2	2	2	
	σ	42	19	1	1	

...continued

[a]Copper values are given in ug Cu/l.

Table 8. (Continued)

SUMMARY OF FISH SURVIVAL DATA AND COPPER MEASUREMENT[a]

	Statistic	Total Copper	Soluble Copper	Particulate Copper	Free & Labile Copper	Percentage Survival
Bioassay B	$\bar{X}$	<1	---	---	---	97.0
(pH 7.00)	N	1	---	---	---	
	s	---	---	---	---	
	$\bar{X}$	39	27	16	10	92.5
	N	2	2	2	2	
	s	12	9	7	4	
	$\bar{X}$	62	40	26	15	76.5
	N	2	2	2	2	
	s	3	8	2	0	
	$\bar{X}$	155	104	54	71	50.0
	N	3	2	2	2	
	s	18	40	10	38	
	$\bar{X}$	254	183	68	96	33.0
	N	3	2	2	2	
	s	49	74	34	56	
	$\bar{X}$	340	244	95	109	13.0
	N	3	2	2	2	
	s	46	12	18	58	

...continued

[a]Copper values are given in ug Cu/l.

Table 8. (Continued)

SUMMARY OF FISH SURVIVAL DATA AND COPPER MEASUREMENT[a]

	Statistic	Total Copper	Soluble Copper	Particulate Copper	Free & Labile Copper	Percentage Survival
Bioassay C	$\bar{X}$	<1	---	---	---	100.0
	N	1	---	---	---	
	s	---	---	---	---	
	$\bar{X}$	39	---	---	---	97.0
	N	4	---	---	---	
	s	5	---	---	---	
	$\bar{X}$	70	---	---	---	79.5
	N	4	---	---	---	
	s	8	---	---	---	
	$\bar{X}$	171	---	---	---	61.5
	N	3	---	---	---	
	s	7	---	---	---	
	$\bar{X}$	284	---	---	---	46.5
	N	4	---	---	---	
	s	14	---	---	---	
	$\bar{X}$	450	---	---	---	28.0
	N	4	---	---	---	
	s	26	---	---	---	

[a]Copper values are given in ug Cu/l.

In bioassays A and B total, soluble, particulate, free, and labile copper were measured. However, only total copper was determined in bioassay C because at pH 5.67 total copper was equal to soluble copper, hence particulate copper was absent. In addition, total copper at this pH is equal to free and labile copper.

The performance characteristics of the bioassay experiments substantiate the validity of the newly applied techniques of chemical controls. Results of bioassays reported in Table 8 need further validation due to lack of replications and should not be used as fish toxicity data per se.

REFERENCES

1. Stumm, W., and H. Bilinski. Trace Metals in Natural Waters: Difficulties of Interpretation Arising from our Ignorance on their Speciation. In: Advances in Water Pollution Research: Proceedings of the 6'ath International Conference held in Jerusalem, June 18-23, 1972, S.H. Jenkins (ed.). Oxford, England, Pergamon Press, 1973. p. 39-49.

2. Duursma, E.K., and W. Sevenhuysen. Note on Chelation and Solubility of Certain Metals at Different pH Values. Nethl. J. Sea Res. 3:95-106, 1966.

3. Siegel, A., and R. Burke. Sorption Studies of Cations on 'Bubble Produced Organic Aggregates' in Sea Water. Deep Sea Research. 12:789, 1965.

4. Bayer, E. Structure and Specificity of Organic Chelating Agents. Angew. Chem. 3:325, 1964.

5. Eriksson, I., and T. Svedberg. The Molecular Weights and pH-Stability Regions of the Hemocyanins. Biol. Bull. 71:498, 1936.

6. Gurd, F.R.N. Chemical Specificity in Biological Interactions. New York, Academic Press, 1954.

7. Goldberg, E.D. Minor Elements in Sea Water. In: Chemical Oceanography, J.P. Riley and G. Skirrow (eds.). New York, Academic Press, 1965. p. 163-196.

8. Hood, D.W. (ed.) Organic Matter in Natural Waters. Institute of Marine Sciences, University of Alaska, 1970. Vol. 1.

9. Corcoran, E.F., and J.E. Alexander. The Distribution of Certain Trace Elements in Tropical Sea Water and Their Biological Significance. Bull. Marine Sci. Gulf Carribbean. 14:594-602, 1964.

10. Slowey, J.F., and D.W. Hood. The Chemistry and Analysis of Trace Metals in Sea Water. Ann. Rept. AEC-Contract No. AT(40-1)-2799, Texas A&M Rept. 66-24. College Station, Texas, 1966.

11. Slowey, J.F., and L.M. Jeffrey. Evidence for Organic Complexed Copper in Sea Water. Nature, 215:377-78, 1967.

12. Williams, P.M. The Association of Copper with Dissolved Organic Matter in Sea Water. Limnol. Oceanogr. 14:136-38, 1969.

13. Koshy, E., and A.K. Ganguly. Organic Materials in the Marine Environments and Their Interactions with Some Heavy Metals. AEC, Bhabha At. Res. Cent. India. B.A.R.C.-402, 1969. 111 p.; Chem. Abstr. 72:82826, 1970.

14. Koshy, E., N.V.M. Desai, and A.K. Ganguly. Organometallic Interactions in the Marine Environment. I. Interaction of Some Metallic Ions with Dissolved Organic Substances in Sea Water. Curr. Sci. 38:555, 1969.

15. Duursma, E.K. Organic Chelation of ^{60}Co and ^{65}Zn by Leucine in Relation to Sorption by Sediments. In: Proceedings of the Symposium on Organic Matter in Natural Waters, D.W. Hood (ed.), Institute of Marine Sciences, University of Alaska, Occa. Pub. 1:387-97, 1970.

16. Barsdate, R.J., and W.R. Matson. Trace Metals in Arctic and Sub-Arctic Lakes with Reference to the Organic Complexes of Metals. In: International Symposium on Radioecological Concentration Processes, B. Aberg and F.P. Hungate (eds.). New York, Pergamon Press, 1966. p. 711-19.

17. Matson, W.R., and D.K. Roe. Trace Metal Analysis of Natural Media by Anodic Stripping Voltammetry. Anal. Instrum. 4:19-22, 1966.

18. Allen, H.E., W.R. Matson, and K.H. Mancy. Trace Metal Characterisation in Aquatic Environments by Anodic Stripping Voltammetry. J. Water Poll. Control Fed. 42:573-81, 1970.

19. Barsdate, R.J. Transition Metal Binding by Large Molecules in High Latitude Waters. In: Proceedings of the Symposium on Organic Matter in Natural Waters, D.W. Hood (ed.). Institute of Marine Sciences, University of Alaska, Occa. Pub. 1:485-93, 1970.

20. DeKock, P.C. Influence of Humic Acids on Plant Growth. Science. 121:473, 1955.

21. Prakash, A., and M.A. Rashid. Influence of Humic Substances on the Growth of Marine Phytoplankton: Dinoflagellates. Limnol. Oceanogr. 13:598, 1968.

32. Johnston, R. Sea Water, The Natural Medium of Phytoplankton. II. Trace Metals and Chelation, and General Discussion. J. Mar. Biol. Ass. U.K. 44:87-109, 1964.

33. Jones, G.E. Effect of Chelating Agents on the Growth of Escherichia coli in Sea Water. J. Bact. 87:483, 1964.

34. Rather, R.T., and J.H. Ryther. Organic Chelators: Factors Affecting Primary Production in the Cromwell Current Upwelling. J. Exp. Mar. Biol. Ecol. 3:191, 1969.

35. Erickson, B.J., F.E. Maloney, and J.H. Gentile. Effect of Nitrilotriacetic Acid on the Growth and Metabolism of Estuarine Phytoplankton. J. Water Poll. Control Fed. 42:R329, 1970.

36. Stumm, W., and J.J. Morgan. Aquatic Chemistry. New York, Wiley-Interscience, 1970. 583 p.

37. Cairns, J., Jr., and A. Scheier. The Effects of Temperature and Hardness of Water Upon the Toxicity of Zinc to the Common Bluegill (Lepomis macrochirus Raf.). Notul. Nat. 299:12, 1957.

38. Wurtz, C. Zinc Effects on Fresh Water Molluska. Nautilus. 76:53, 1962.

39. Pickering, Q.H., and C. Henderson. The Acute Toxicity of Some Heavy Metals to Different Species of Warm Water Fishes. Int Proceedings of the 19th Purdue Industrial Waste Conf., Purdue, Ind., 1963.

40. Mount, D.I. The Effect of Total Hardness and pH on Acute Toxicity of Zinc to Fish. Air and Water Pollut. Int. J. 10:49-56, 1967.

41. Chen, C.W. A Kinetic Model of Fish Toxicity Threshold. Ph.D. Thesis. University of California, Berkeley, 1968.

42. McKee, J.E., and H.W. Wolf. Water Quality Criteria. Pub. No. 3-A. The Resources Agency of California State Water Quality and Control Board, 1963. p. 170-71.

43. Lloyd, R. Toxicity of Zinc Sulfate to Rainbow Trout. Ann. App. Biol. 48:84-94, 1960.

44. Fitzgerald, G.P. Factors Affecting the Toxicity of Copper to Algae and Fish. Amer. Chem. Soc., Div. of Waste Water Chemistry, 1953. p. 21-24.

45. Grande, M. Effect of Copper and Zinc on Salmonid Fishes. Proceedings of the 3rd Int. Conf. on Water Poll. Res., Munich. 1:97-111, 1966.

36. Nielsen, E.S., and A. Wium-Andersen. Copper Ions as Poisons in the Sea and in Freshwater. Mar. Biol. 6:93-97, 1970.

37. Pauli, F.W. Some Recent Developments in Biogeochemical Research. Proceedings of the Geol. Ass. Canada. 19:45-49, 1968.

38. Hynes, H.B.N. The Biology of Polluted Waters. Liverpool, England, Liverpool University Press, 1960. p. 73-76.

39. Lazrus, A.L., E. Lorange, and J.P. Lodge. Lead and Other Metal Ions in United States Precipitation. Environ. Sci. Technol. 4: 55-58, April 1970.

40. Kramer, J.R. Fate of Atmospheric Sulfur Dioxide and Related Substances as Indicated by the Chemistry of Precipitation. Dept. of Geology, McMaster University, Hamilton, Ontario. Ministry of Environment, Air Management Branch, June 1973. p.18-36.

41. Control of Potential Sources of Pollution Due to Proposed Copper Mining Operations in Puerto Rico. Arcadia, Calif., Engineering-Science, Inc., 1967. 118 p.

42. Sheffer, H.W., and L.G. Evans. Copper Leaching Practices in the Western United States. Information Circular 8341. Washington, DC, U.S. Dept. Interior, Bureau of Mines, 1968.

43. Prokovich, N.P. Siltation and Pollution Problems in Spring Creek, Shasta County, California. J. Amer. Water Works Ass. 57:986-95, 1965.

44. Moore, G.T., and K.F. Kellerman. A Method of Destroying or Preventing the Growth of Algae and Certain Pathogenic Bacteria in Water Supplies. Bureau of Plant Industry Bulletin 64. Washington, DC, U.S. Dept. Agr., 1904. p. 9-44.

45. Goudey, R.F. A New Method of Copper Sulfating Reservoirs for Control of Microscopic Organisms. M.S. Thesis. Los Angeles, University of Southern California, 1935.

46. Nichols, M.S., T. Henkel, and D. McNall. Copper in Lake Muds from Lakes of the Madison Area. Trans. Wis. Acad. Sci. Arts. Lett. 38:333-50, 1946.

47. Sanches-Vasques, I. Aqueous Environmental Chemistry of Copper. Ph.D. Thesis. University of Wisconsin, Madison, 1971.

48. Schnitzer, M., and I. Hoffman. Thermogravimetric Analysis of the Salts and Metal Complexes of a Soil Fulvic Acid. Geochim. Cosmochim. Acta. 31:7-15, 1967.

49. Riley, G.A. Limnological Studies in Connecticut. Part II. The Copper Cycle. Ecol. Monogr. 9:166-94, 1939.

50. Corcoran, E.F. Bulletin of Marine Science of the Gulf and Caribbean. 15(4):594, 1964.

51. Hume, D.N. Analysis of Water for Trace Metals, Present Capabilities and Limitations. In: Equilibrium Concepts in Natural Water Systems, R.F. Gould (ed.). Washington, D.C., American Chemical Society, 1967. p. 30-44.

52. Nancy, K.H. Analytical Problems in Water Pollution Control. In: National Bureau of Standards Special Publication 351, Proceedings of the 24th Annual Summer Symposium, 1971, p. 297-382.

53. Morrison, G.H. (ed.). Trace Analysis, Physical Methods. New York, Interscience Publishers, 1965. 582 p.

54. Foster, P., and A.W. Morris. The Seasonal Variation of Dissolved Ionic and Organically Associated Copper in the Menai Straits. Deep Sea Res. 18:231-36, 1971.

55. Kahn, H.L. Principles and Practice of Atomic Absorption. In: Trace Inorganics in Water; A Symposium sponsored by the Division of Water, Air, and Waste Chemistry at the 153rd Meeting of the American Chemical Society, Miami Beach, Fla., April 10-13, 1967. Washington, D.C., American Chemical Society, Advances in Chemistry Series 73, 1968 p. 183-229.

56. Osteryoung, J.G., and R.A. Osteryoung. Pulse Polarographic Analysis of Toxic Heavy Metals. Amer. Lab. 9:8-11, 1977.

57. Standard Methods for the Examination of Water and Wastewater. 12th Ed. New York, American Public Health Association, Inc., 1965. 769 p.

58. Atkins, D.H.F., and A.A. Smales. Activation Analysis. Advan. Inorg. Chem. Radiochem. 1:315-45, 1959.

59. Kowalski, B.R., T.L. Isenhour, and R.E. Seavers. Ultra-Trace Mass Spectrometric Metal Analysis Using Heptafluorodimethyloctanedione. Anal. Chem. 41:998-1003, 1969.

60. Guilbault, G.G., and T. Heisel. Determination of Mixtures of Copper II and Manganese II by Electron Spin Resonance. Anal. Chem. 41:1100-03, 1969.

61. Hetman, J. Determination of Traces of Cyanide in Water by Cathode Ray Polarography. J. Appl. Chem. 10:16-18, January 1960.

62. Pohl, F.A. Zur Schnellbestimmung von Metallspuren in Reaktorabwässern. Z. Anal. Chem. 197(2):193-97, 1963.

63. Whitnack, G.C. Applications of Cathode-Ray Polarography in the Field of Oceanography. J. Electroanal. Chem. 2(2):110-15, March/April 1961.

64. Schmidt, H., and M. von Stackelberg. Modern Polarographic Methods. New York, Academic Press, 1961.

65. Matson, W.R., H.E. Allen, and F. Rakshan. Trace Metal Organic Complexes in the Great Lakes. Presented at the 157th National American Chemical Society Meeting, Minneapolis, Minn., April 1969.

66. Barsdate, R.J., and W.R. Matson. Trace Metals in Arctic and Sub-Arctic Lakes with Reference to the Organic Complexes of Metals. In: International Symposium on Radioecological Concentration Processes, B. Aberg and F.P. Hungate (eds.). New York, Pergamon Press, 1966. p. 711-19.

67. Bender, M.E., W.R. Matson, and R.A. Jordan. On the Significance of Metal Complexing Agents in Secondary Sewage Effluents. Environ. Sci. Technol. 4:520-21, 1970.

68. Zirino, A.R., and M.L. Healy. Inorganic Zinc Complexes in Seawater. Limnol. Oceanogr. 15:956-58, 1970.

69. Peterson, T.L., D.O. Brant, and E.H. Nancy. Characterization of Trace Metals in Municipal Water Supplies by Thin Layer Anodic Stripping Voltammetry. Presented at the 160th National American Chemical Society Meeting, Chicago, Ill., September 1970.

70. Barendicht, E. Stripping Voltammetry. In: Electroanalytical Chemistry, A.J. Bard (ed.). New York, Marcel Dekker, Inc., 1967. p. 53-109.

71. Kodama, H., and T. Noda. Application of a Mercury-Coated Platinum Electrode to the A.C. Stripping Analysis of a Trace Amount of Metal Ions. Bull. Chem. Soc. Jap. 42:2699-701, 1969.

72. Hirva, T., S. Oki, and A. Misuike. Anodic Stripping Voltammetry of Lead and Cadmium Using Radiofrequency Technique. Bunseki Kagaku. 18:1406-07, 1969; Chem. Abstr. 72:74431, 1970.

73. Kemula, W. Application of Stripping Processes in Voltammetry. Pure Appl. Chem. 21:449-60, 1970.

74. Christian, G.D. Anodic Stripping Pulse Voltammetry. J. Electroanal. Chem. Interfacial Electrochem. 23:1-7, 1969.

75. Matson, W.R., D.E. Roe, and D.E. Carritt. Composite Graphite-Mercury Electrode for Anodic Stripping Voltammetry. Anal. Chem. 37: 1594-95, 1965.

76. Roe, D.K., and J. Toni. An Equation for Anodic Stripping Curves of Thin Mercury-Film Electrodes. Anal. Chem. 37:1503-06, 1965.

77. O'Shea, T.A. Anodic Stripping Voltammetric Study of the Competitive Interactions Between Trace Metals and the Alkaline Earths for Complexing Ligands in Aquatic Environments. Ph.D. Thesis, The University of Michigan, Ann Arbor, 1972.

78. Lingane, J.J. Electroanalytical Chemistry. 2nd Ed. New York, Interscience Publishers, Inc., 1958. p. 316.

79. Matson, W.R. Trace Metals, Equilibria, and Kinetics of Trace Metal Complexes in Natural Media. Ph.D. Thesis. Massachusetts Institute of Technology, Cambridge, 1968.

80. Keith, J.F. The Solubility of Malachite. Can. J. Chem. 35: 1332-40, 1957.

81. Gilman, J. Reported in Stability Constants by L.G. Sillen and A.E. Martell. Special Publication No. 17, Chemical Society, London, 1964.

82. Stiff, M.J. Copper/Bicarbonate Equilibria in Solutions of Bicarbonate Ion Concentrations Similar to Those Found in Natural Waters. Water Res. 5:171-76, 1971.

83. Kielland, J. Individual Activity Coefficients of Ions in Aqueous Solution. J. Amer. Chem. Soc. 59:1675-78, 1937.

84. Sillen, L.G. and A.E. Martell. Reported in Stability Constants by L.G. Sillen and A.E. Martell. Special Publication No. 17, Chemical Society, London, 1964.

85. Black, J.A., R.F. Roberts, D.M. Johnson, D.D. Minicucci, E.H. Nancy, and H.E. Allen. The Significance of Physical Variables in Aquatic Bioassays of Heavy Metals. In: Bioassay Techniques and Environmental Chemistry, G.E. Glass (ed.). Ann Arbor, Mich., Ann Arbor Science Publishers, Inc., 1972.

86. Roberts, R.F., and H.E. Allen. The Control of pH and Total Alkalinity or Total Carbonate in Aquatic Bioassays. Trans. Amer. Fish. Soc. 101(4):732-36, October 1972.

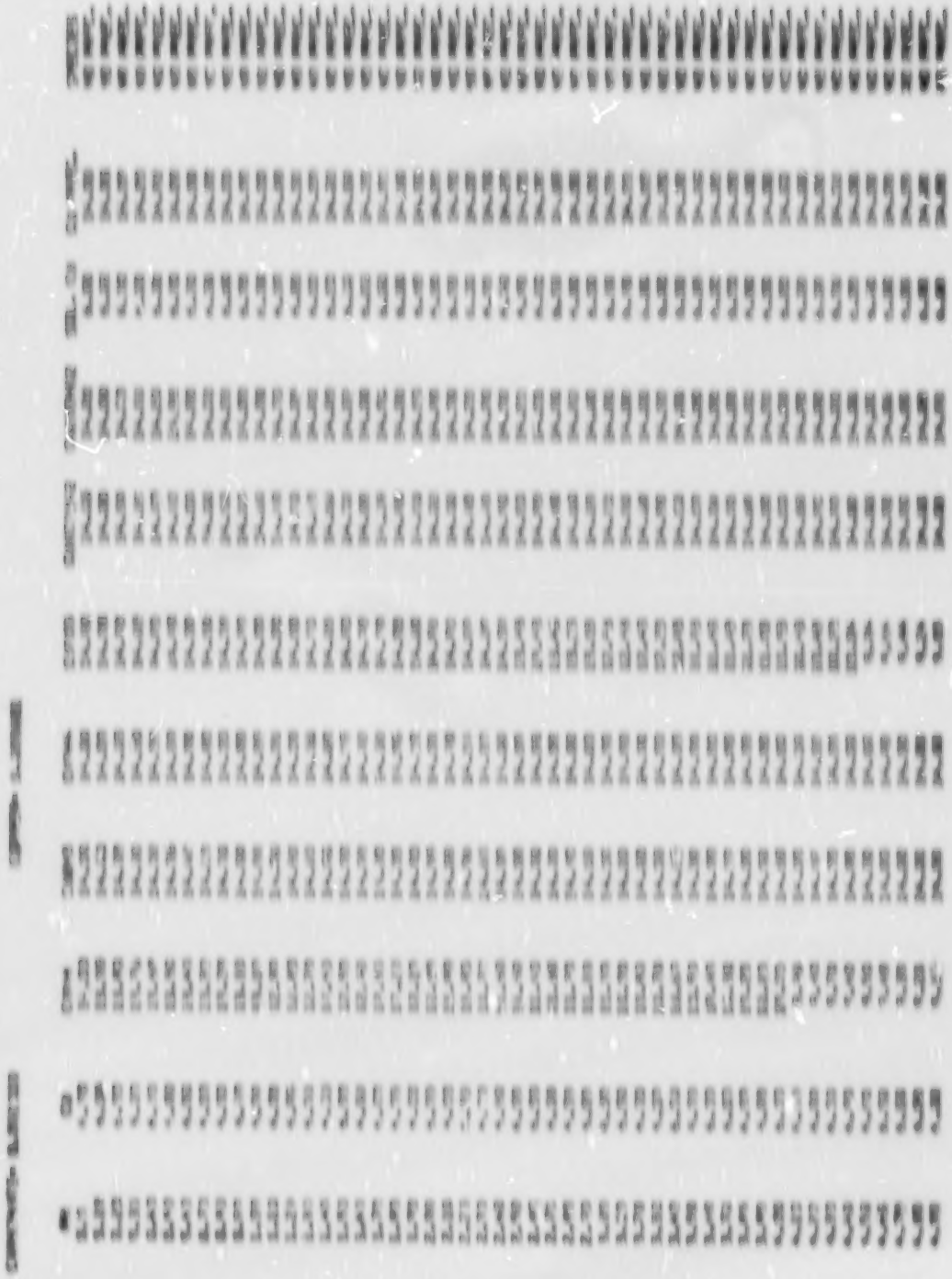
87. Ernst, W., H.E. Allen, and K.H. Mancy. Characterization of Trace Metal Species and Measurement of Trace Metal Stability Constants by Electrochemical Techniques. Water Res. 9:969-79, 1975.

88. Mount, D.I., and R.E. Warner. A Serial-Dilution Apparatus of Continuous Delivery of Various Concentrations of Materials in Water. U.S. Public Health Service Publication No. 999-WP-23, Washington, D.C., 1965.

89. Eaves, L.A., and J.D. Conners. Continuous-Flow Fish Bi-assay Apparatus for Municipal and Industrial Effluents. Sanitary Engineering Research Laboratory, SERL Report No. 71-3, University of California, Berkeley, 1971.

APPENDIX A

AQUEOUS COPPER EQUILIBRIA AT CONSTANT TOTAL CARBONATE AND VARIABLE pH VALUES



APPENDIX B

COMPUTATION OF EQUILIBRIUM pH AND TOTAL ALKALINITY AT DIFFERENT PARTIAL PRESSURES OF CARBON DIOXIDE

PRESS (ATM)	WATER MG/L	FAULT CACO3 MG/L	[illegible]	[illegible]	[illegible]	ALKALINITY EQ/L	HARDNESS CACO3 MG/L	TOTAL IONIC [illegible]
0.147E-02	12.387	10	0.301E-04	0.179E-03	0.100E-06	0.179E-03	0.501E-01	0.179E-03
0.295E-02	24.775	20	0.100E-03	0.599E-03	0.397E-06	0.599E-03	0.247E-01	0.599E-03
0.443E-02	37.162	30	0.210E-03	0.998E-03	0.100E-05	0.599E-03	0.247E-01	0.599E-03
0.591E-02	49.550	40	0.397E-03	0.798E-03	0.397E-06	0.798E-03	0.327E-01	0.798E-03
0.739E-02	61.938	50	0.291E-03	0.998E-03	0.503E-06	0.998E-03	0.397E-01	0.998E-03
0.887E-02	74.325	60	0.300E-03	0.119E-02	0.695E-06	0.119E-02	0.495E-01	0.119E-02
0.103E-01	86.713	70	0.350E-03	0.139E-02	0.701E-06	0.139E-02	0.707E-01	0.139E-02
0.118E-01	99.100	80	0.403E-03	0.159E-02	0.403E-06	0.159E-02	0.661E-01	0.159E-02
0.133E-01	111.489	90	0.461E-03	0.179E-02	0.403E-06	0.179E-02	0.594E-01	0.179E-02
0.147E-01	123.876	100	0.501E-03	0.199E-02	0.100E-05	0.199E-02	0.703E-01	0.199E-02
0.162E-01	136.263	110	0.551E-03	0.219E-02	0.110E-05	0.219E-02	0.675E-01	0.219E-02
0.177E-01	148.651	120	0.601E-03	0.239E-02	0.120E-05	0.239E-02	0.827E-01	0.239E-02
0.192E-01	161.039	130	0.651E-03	0.259E-02	0.130E-05	0.259E-02	0.389E-01	0.259E-02
0.207E-01	173.426	140	0.701E-03	0.279E-02	0.140E-05	0.279E-02	0.347E-01	0.279E-02
0.221E-01	185.814	150	0.751E-03	0.299E-02	0.150E-05	0.299E-02	0.354E-01	0.299E-02
0.236E-01	198.201	160	0.801E-03	0.319E-02	0.160E-05	0.319E-02	0.321E-01	0.319E-02
0.251E-01	210.589	170	0.851E-03	0.339E-02	0.170E-05	0.339E-02	0.274E-01	0.339E-02
0.266E-01	222.977	180	0.901E-03	0.359E-02	0.180E-05	0.359E-02	0.279E-01	0.359E-02
0.281E-01	235.364	190	0.952E-03	0.379E-02	0.190E-05	0.379E-02	0.254E-01	0.379E-02
0.295E-01	247.752	200	0.101E-02	0.399E-02	0.200E-05	0.399E-02	0.247E-01	0.399E-02
0.310E-01	260.139	210	0.107E-02	0.419E-02	0.210E-05	0.419E-02	0.239E-01	0.419E-02
0.325E-01	272.527	220	0.112E-02	0.439E-02	0.220E-05	0.439E-02	0.227E-01	0.439E-02
0.340E-01	284.915	230	0.117E-02	0.459E-02	0.230E-05	0.459E-02	0.217E-01	0.459E-02
0.355E-01	297.302	240	0.123E-02	0.479E-02	0.240E-05	0.479E-02	0.210E-01	0.479E-02
0.369E-01	309.690	250	0.125E-02	0.499E-02	0.250E-05	0.499E-02	0.199E-01	0.499E-02
0.384E-01	322.078	260	0.134E-02	0.519E-02	0.260E-05	0.519E-02	0.192E-01	0.519E-02
0.399E-01	334.465	270	0.139E-02	0.539E-02	0.270E-05	0.539E-02	0.185E-01	0.539E-02
0.414E-01	346.853	280	0.140E-02	0.559E-02	0.280E-05	0.559E-02	0.178E-01	0.559E-02
0.429E-01	359.240	290	0.145E-02	0.579E-02	0.290E-05	0.579E-02	0.172E-01	0.579E-02
0.443E-01	371.628	300	0.150E-02	0.599E-02	0.300E-05	0.599E-02	0.167E-01	0.599E-02

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
0.12700E-04	12.587	10	0.41.35E-08	0.17.75E-03	0.84.45E-05	0.15.48E-03	0.57.65E-03	0.37.96E-03
0.28.75E-04	24.775	20	0.49.56E-08	0.35.61E-03	0.17.76E-04	0.38.79E-03	0.20.56E-03	0.40.73E-03
0.38.71E-04	37.267	30	0.13.95E-05	0.53.57E-03	0.26.86E-04	0.59.84E-03	0.38.44E-03	0.40.92E-03
0.53.17E-04	49.950	40	0.19.71E-05	0.71.79E-03	0.35.85E-04	0.79.47E-03	0.38.44E-03	0.40.92E-03
0.66.63E-04	61.939	50	0.22.57E-05	0.89.98E-03	0.45.07E-04	0.99.17E-03	0.11.12E-03	0.17.94E-03
0.80.09E-04	74.325	60	0.27.14E-05	0.10.89E-02	0.54.07E-04	0.11.91E-02	0.13.79E-03	0.17.94E-03
0.93.56E-04	86.713	70	0.31.70E-05	0.12.62E-02	0.63.27E-04	0.13.84E-02	0.15.79E-03	0.17.94E-03
0.10.70E-03	99.170	80	0.36.26E-05	0.14.47E-02	0.72.39E-04	0.15.98E-02	0.17.83E-03	0.17.94E-03
0.12.04E-03	111.489	90	0.40.82E-05	0.16.27E-02	0.81.59E-04	0.17.98E-02	0.19.78E-03	0.17.94E-03
0.13.39E-03	123.476	100	0.45.39E-05	0.18.08E-02	0.90.79E-04	0.19.97E-02	0.21.73E-03	0.17.94E-03
0.14.74E-03	136.263	110	0.49.96E-05	0.19.88E-02	0.99.99E-04	0.21.97E-02	0.23.68E-03	0.17.94E-03
0.16.09E-03	148.651	120	0.54.53E-05	0.21.71E-02	0.10.91E-03	0.23.97E-02	0.25.63E-03	0.17.94E-03
0.17.45E-03	161.139	130	0.59.07E-05	0.23.51E-02	0.11.74E-03	0.25.97E-02	0.27.57E-03	0.17.94E-03
0.18.77E-03	173.426	140	0.63.63E-05	0.25.31E-02	0.12.66E-03	0.27.97E-02	0.29.52E-03	0.17.94E-03
0.20.12E-03	185.814	150	0.68.19E-05	0.27.14E-02	0.13.58E-03	0.29.97E-02	0.31.47E-03	0.17.94E-03
0.21.47E-03	198.201	160	0.72.75E-05	0.28.94E-02	0.14.51E-03	0.31.97E-02	0.33.41E-03	0.17.94E-03
0.22.81E-03	210.589	170	0.77.31E-05	0.30.74E-02	0.15.42E-03	0.33.96E-02	0.35.36E-03	0.17.94E-03
0.24.16E-03	222.977	180	0.81.87E-05	0.32.55E-02	0.16.34E-03	0.35.96E-02	0.37.31E-03	0.17.94E-03
0.25.51E-03	235.364	190	0.86.44E-05	0.34.35E-02	0.17.26E-03	0.37.96E-02	0.39.25E-03	0.17.94E-03
0.26.85E-03	247.752	200	0.91.00E-05	0.36.15E-02	0.18.17E-03	0.39.96E-02	0.41.20E-03	0.17.94E-03
0.28.20E-03	260.139	210	0.95.56E-05	0.37.96E-02	0.19.09E-03	0.41.95E-02	0.43.15E-03	0.17.94E-03
0.29.54E-03	272.527	220	0.10.01E-04	0.39.76E-02	0.19.99E-03	0.43.95E-02	0.45.09E-03	0.17.94E-03
0.30.89E-03	284.915	230	0.10.46E-04	0.41.57E-02	0.20.90E-03	0.45.95E-02	0.47.04E-03	0.17.94E-03
0.32.24E-03	297.302	240	0.10.92E-04	0.43.37E-02	0.21.81E-03	0.47.95E-02	0.48.99E-03	0.17.94E-03
0.33.58E-03	309.690	250	0.11.38E-04	0.45.18E-02	0.22.72E-03	0.49.94E-02	0.50.93E-03	0.17.94E-03
0.34.93E-03	322.078	260	0.11.83E-04	0.46.98E-02	0.23.63E-03	0.51.94E-02	0.52.88E-03	0.17.94E-03
0.36.27E-03	334.465	270	0.12.29E-04	0.48.79E-02	0.24.55E-03	0.53.94E-02	0.54.83E-03	0.17.94E-03
0.37.62E-03	346.853	280	0.12.75E-04	0.50.59E-02	0.25.46E-03	0.55.94E-02	0.56.78E-03	0.17.94E-03
0.38.97E-03	358.240	290	0.13.20E-04	0.52.39E-02	0.26.36E-03	0.57.94E-02	0.58.72E-03	0.17.94E-03
0.40.31E-03	371.628	300	0.13.66E-04	0.54.20E-02	0.27.27E-03	0.59.94E-02	0.60.67E-03	0.17.94E-03

[illegible]	[illegible]		[illegible]					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]